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PANCREATIC ISLET ALLOGRAFT IMMUNOGENICITY IN IMMUNITY AND TOLERANCE

©

BY
MARILYNE G. COULOMBE

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
IN
MEDICAL SCIENCES (MEDICINE)

EDMONTON, ALBERTA

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This work was performed at the
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This text is dedicated to
my family
for their never-ending love and support

and to Steve
for his unfailing friendship

ABSTRACT

The nature of tolerance induction to extrathymic antigens, in particular, the fate of potentially reactive T cells upon encounter with non-immunogenic tissues in the periphery, is controversial. One intriguing proposition is that presentation of antigen (signal 1) without the delivery of an appropriate costimulator (signal 2) required for T cell activation results in the functional inactivation of the reactive T cell. This form of antigen presentation is represented by APC-depleted pancreatic islet allografts. Such grafts were used to determine whether signal 1 antigen presentation leads to T cell deletion/inactivation in either mature adult, or newly developed, immune systems.

C57BL/6 (B6, H-2^b) pancreatic islets were depleted of APCs by 95% O_2 culture prior to transplantation. Such cultured islets reverse diabetes indefinitely in nonimmunosuppressed, chemically induced diabetic BALB/c or Igh congenic immuno-incompetent C.B-17*scid* (scid) (H-2^d) recipients. Although adult BALB/c recipients can reject APC-depleted allografts when immunized with B6 APCs early after grafting, they become resistant to such immunization with time after grafting. This resistance to rejection is associated with tolerance induction in that: 1) Such animals also resist rejection of secondary B6 islet grafts and 2) Spleen cells from tolerant animals transferred to scid mice confer the ability of these animals to reject third-party, but not donor-type, cultured islets. Analysis of anti-donor reactivity in tolerant animals revealed normal responses - including tissue (islet) specific reactivity - both *in vitro* and *in vivo*. This indicated that tolerance was not due to any apparent deletion/inactivation of donor-reactive T cells.

To examine whether newly developing T cells become tolerant to APC-depleted allografts, scid mice were reconstituted with T cell-depleted BALB/c bone marrow after islet grafting. Reconstituted mice became immunocompetent and remained normoglycemic. Despite T cell maturation in the presence of the allograft, immunization with donor-type APCs triggered acute graft rejection. This finding indicated that T cells emerging from the thymus were neither activated nor tolerized by the established peripheral allograft.

Taken together, these studies indicate that APC-depleted allografts do not directly tolerize donor-reactive T cells. Thus, cultured islet allografts, a model of 'signal 1' antigen presentation, do not lead to the clonal deletion or inactivation of mature or newly developed donor-specific T cells. Rather, it is proposed that, in the adult animal, tolerance to cultured allografts involves an active mechanism which regulates the function of donor-reactive T cells *in vivo*.

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TABLE OF CONTENTS

I: INTRODUCTION PAGE

PANCREATIC ISLET TRANSPLANTATION 1

HISTORICAL PERSPECTIVE OF SELF-NONSELF DISCRIMINATION 3
  Instructional theories 4
  Selection theories 5
  The classical concept of alloreactivity: the transplantation paradox 7
  Two signal models for lymphocyte induction: the Bretscher/Cohn model 10
  The two signal model for T cell activation 11
  Experimental support for the two signal model 12
  Implication of the two-signal hypothesis: the stimulator cell model 13
  Implication of the stimulator cell model: modification of tissue immunogenicity 14
  Antigenicity versus immunogenicity of MHC alloantigens 16
ALLOGRAFT TOLERANCE 20
  Definitions of tolerance 21
    Clonal deletion 21
    Clonal anergy/inactivation 22
    Suppression/regulation 22
INDUCTION OF TOLERANCE 23
  Site of tolerance induction: thymus versus periphery 23
    Thymic environment 23
    Peripheral environment 26
  Stage of lymphocyte activation 27
  Nature of antigen presentation 29
    Signal one alone induces tolerance 29
    T cell indifference to peripheral self antigen 32
    Tolerance and tissue immunogenicity 33
MAINTENANCE OF TOLERANCE 34
  Passive Mechanisms of Tolerance 35
    Clonal Deletion 35
    Clonal Inactivation/Anergy 36

PAGE

Active Mechanisms of Tolerance 37
    Active mechanisms maintaining tolerance to self 37
    Active mechanisms in allograft tolerance 38
TOLERANCE TO APC-DEPLETED ALLOGRAFTS 39
HYPOTHESIS 43
REFERENCES 44

II. TOLERANCE INDUCTION TO CULTURED ISLET ALLOGRAFTS

Characterization of the Tolerant State 61
Materials and Methods 63
Results 65
Discussion 69
References 81

III. TOLERANCE INDUCTION TO CULTURED ISLET ALLOGRAFTS

Status of Anti-Donor Reactivity in Tolerant Animals 84
Materials and Methods 85
Results 89
Discussion 93
References 105

IV. LACK OF T CELL TOLERANCE TO EXTRATHYMIC ALLOANTIGEN 109
Materials and Methods 110
Results 113
Discussion 116
References 126

V. DISCUSSION 129
Antigenicity versus immunogenicity 129
Long-term allograft acceptance versus tolerance 130
Experimental variables which influence graft stabilization 131
Adaptation of the islet allograft does not account for graft stabilization 133

PAGE

Mechanism of tolerance to APC-depleted grafts (active versus passive) 134
Assessment of anti-donor reactivity *in vitro* 135
Assessment of anti-donor reactivity *in vivo* 136
Tissue-specificity of the tolerant state 137
Lack of T cell specificity to an islet-derived peptide 138
Does 'signal 1' antigen presentation lead to the inactivation of newly developed T cells? 141
Problems with tolerance induction by 'signal 1' antigen presentation 144
Active mechanisms of tolerance 148
Immune deviation in allograft tolerance 151
Proposed mechanism of tolerance to APC-depleted allografts... 151
Speculations of tolerance induction 153
Summary and conclusions 160
References 162

APPENDIX A. The theory of allogeneic reactivity 170
APPENDIX B. Antigenicity versus immunogenicity 174
APPENDIX C. Tissue specificity of the tolerant state 176
APPENDIX D. Materials and Methods 180

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
II-1	Islet allograft stabilization is influenced by both initial mass and the time between grafting and donor-type APC challenge.	73
II-2	Secondary donor-type islet grafts are protected in recipients bearing cultured allografts.	74
II-3	Long-term residence of a cultured islet allograft is not necessary or sufficient for graft stabilization.	75
II-4	Sentinel islet allograft survival in scid mice reconstituted with spleen cells from tolerant or age-matched control mice.	76
II-5	Survival of thyroid allografts in recipients of long-term cultured islet allografts.	77
III-1	CTLp frequencies of lymph node and spleen cell populations from tolerant and age-matched control animals.	97
III-2	Survival of established donor (B6) or syngeneic (BALB/c) sentinel islet grafts following transfer of *in vitro* activated (BALB/c anti-B6) T cells derived from tolerant or control BALB/c mice.	98
IV-1	FACS analysis of peripheral blood lymphocyte populations in naive BALB/c, C.B-17 scid and scid mice reconstituted with BALB/c bone marrow.	120
IV-2	C.B-17scid mice are capable of rejecting B6 (H-2^b) thyroid allografts eight weeks after reconstitution with BALB/c (H-2^d) bone marrow cells.	121

TABLE	DESCRIPTION	PAGE
IV-3	Survival of APC-depleted C57BL/6 ($H-2^b$) islet allografts in C.B-17scid ($H-2^d$) mice following maturation of the immune system.	122
B-1	Effect of IFN-γ treatment on the survival of cultured islet allografts.	175
C-1	Analysis of T cell subsets in peripheral blood of untreated or antibody-treated BALB/c ($H-2^d$) recipients of B6 ($H-2^b$) thyroid allografts.	177
C-2	B6 ($H-2^b$) thyroid allograft survival in untreated or anti-T cell antibody-treated BALB/c ($H-2^d$) recipients.	177
C-3	Survival of cultured B6 or CBA islet allografts in scid ($H-2^d$) mice after i.p. injection of 'd anti-b' or 'd anti-k' T cells, respectively.	179

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE
II-1	Graft stabilization is a time dependent process	78
II-2	Graft stabilization results in the survival of primary and secondary cultured B6 allografts in BALB/c recipients.	79
II-3	Donor-specific tolerance can be transferred to scid mice.	80
III-1	Lymphoid cell populations from tolerant animals proliferate in response to donor (B6) antigen.	99
III-2	Anti-donor cytotoxic responses of LN cells derived from tolerant or age-matched control animals.	100
III-3	Anti-donor lymphokine production from tolerant animals.	101
III-4	Primed lymphoid cells from tolerant animals secrete IFN-γ in response to donor Class I MHC antigen (EL-4, $H\text{-}2^b$).	102
III-5	*In vitro* activated lymphoid cells derived from tolerant animals are reactive to donor islet antigen.	103
III-6	*In vitro* primed T cells from tolerant animals can mediate donor-specific islet graft destruction *in vivo*.	104
IV-1	Proliferative responses of LN cell populations from BALB/c and bone marrow reconstituted scid mice bearing B6 islet allografts.	123
IV-2	Cytotoxic responses of LN cells from naive BALB/c or bone marrow reconstituted scid mice bearing cultured B6 islet allografts.	124

FIGURE	DESCRIPTION	PAGE
IV-3	APC-depleted B6 islet allografts in C.B-17 scid mice reconstituted with BALB/c bone marrow.	125
V-1	Speculative view of tolerance induction.	154

LIST OF ABBREVIATIONS

ALS	anti-lymphocyte serum
APC	antigen presenting cell
'c'	control molecule
Con A	concanavalin A
CoS	costimulator
CsA	cyclosporin A
CTL	cytotoxic T lymphocyte
CTLp	precursor cytotoxic T lymphocyte
DSBT	donor-specific blood transfusion
DTH	delayed type hypersensitivity
GVH	graft versus host
GP	glycoprotein
IFN-γ	interferon-gamma
IL	interleukin
LCMV	lymphocytic choriomeningitis virus
LN	lymph node
mAb	monoclonal antibody
MHC	major histocompatibility complex
MLC	mixed leucocyte culture
Mls	minor lymphocyte stimulating
RIP	rat insulin promoter
S^+	stimulator cell supplying both signals for T cell activation
S^-	cell which can not supply the second signal
SAg	superantigen
scid	severe-combined immunodeficient
SN	supernatant
SZ	streptozotocin
T'	activated T cell
TCR	T cell receptor
TNF	tumor necrosis factor
UV	ultraviolet
V_β	variable region of the β chain of the T cell receptor

I

INTRODUCTION

PANCREATIC ISLET TRANSPLANTATION

Two decades have passed since the first reports of successful transplantation of pancreatic islets in diabetic rodents (1). Though progress has been slow, the feasibility of pancreatic islet transplantation as a therapy for patients with Type I insulin dependent diabetes mellitus is now apparent. There is a general consensus that good blood glucose control will slow the progression and severity of the debilitating microangiopathic and neurologic complications of this disease (2) and because of this, islet transplantation, as with whole pancreas transplantation, would provide a better alternative to the currently available exogenous insulin therapy. Indeed, it has been demonstrated that transplantation can prevent or reverse early complications of diabetes (3, 4). Islet transplantation has both practical and economic benefits over whole pancreas transplantation. The small mass of the islet graft makes it easy to implant rapidly, safely and economically relative to whole pancreas grafting. Further, post-surgical complications and mortality associated with pancreas grafting would be diminished. Other advantages of islet transplantation include the potential for the establishment of cryopreserved tissue banks (5), the use of immunologically privileged sites (6, 7), and the potential modification of tissue immunogenicity by pretransplant regimens (8, 9) or immunoisolation techniques (10, 11). In addition, the potential to graft xenogeneic islet tissue (12-14) could overcome the shortage of available allogeneic tissue, thus facilitating wide-spread application of this technique. Several of these advantages of islet over pancreas transplantation would preclude the use of long-term immunosuppression and thus extend the patient population eligible for such therapy beyond those with severe vascular and renal complications.

The main objectives of islet transplantation are to graft sufficient quantities of islets to eliminate the need for exogenous insulin therapy, to arrest the development of diabetic complications, and to avoid the long-term use of immunosuppressive agents. The risks associated with long-term immunosuppression (nephrotoxicity, infectious disease, or neoplasia) may outweigh the complications of diabetes mellitus itself. Therefore, the criteria for using immunosuppressive agents for Type I diabetic patients who have not yet developed debilitating complications are more stringent than that for patients suffering from end-stage organ failure since death is not imminent without an islet transplant. Thus, the continuous use of immunosuppressive agents must be avoided.

Recent improvements in technical aspects of islet isolation and purification have led to the application of this technique in the clinic (15) although, to date, clinical islet transplantation has only utilized patients receiving single or multiple organ allografts and who are, as such, heavily immunosuppressed. Several patients without pre-existing diabetes showed extended insulin independence following upper abdominal exenteration and liver-islet replacement (16). Groups in St. Louis (17), Pittsburgh (16), Minnesota (18) and Edmonton (19) have reported significant graft function following intrahepatic human islet transplantation. The Edmonton group has reported that a Type I diabetic patient has been insulin independent for greater than one year after islet allotransplantation (19), however, this type of clinical success in such patients is limited. This limited success may reflect technical failure, allograft rejection or the potential for the original disease to recur and destroy the transplant (20, 21). It is currently unclear which of these factors have contributed to the clinical failures. Thus, there are three major barriers to the clinical success of islet transplantation: 1) availability of donor tissue and the consistent isolation of sufficient quantities of viable islets, 2) allograft rejection and 3) recurrence of the initial disease. Each of these areas require further investigation before islet transplantation becomes available to the diabetic population in general.

This review will focus on the second obstacle to islet allografting mentioned above, the nature of allorecognition leading to graft rejection. As such, a theoretical framework of alloreactivity will be examined with an emphasis on the mechanisms responsible for the induction of donor-specific immunity and tolerance to transplanted tissues. The rationale for inducing tolerance to an allograft is to obviate the need for long-term use of immunosuppressive agents, a particularly important criterion for the clinical application of islet transplantation. Hypotheses developed to explain self-nonself discrimination will be contrasted with those proposed to explain the many empirical observations of induced transplantation tolerance. A clear understanding of the mechanism(s) involved in the induction of tolerance to allografts is necessary if we are to take advantage of this phenomenon in a clinical situation.

HISTORICAL PERSPECTIVE OF SELF-NONSELF DISCRIMINATION

How the immune system distinguishes self from nonself is one of the fundamental questions of immunology. This question has long attracted the attention of immunologists trying to understand how self antigens are distinguished from foreign antigens, how this may relate to autoimmune disease, and from a transplantation point of view, how one can manipulate the immune system to accept foreign tissue as self. Several hypotheses have been proposed to explain immunological tolerance. These hypotheses are generally divided into two main categories: 1) **passive** mechanisms in which antigen specific T cell clones are physically or functionally deleted after interacting with antigen and 2) **active** mechanisms in which the antigen-specific T cell has the potential to react, but is, in some way, regulated by cellular or humoral factors. Passive mechanisms are, thus, *intrinsic* to the antigen-reactive T cell whereas active mechanisms are due to factors *extrinsic* to the reactive T cell. Because the immune repertoire is generated randomly, theoretical models of self-nonself discrimination must account for the control of potential self-reactivity while retaining the ability to respond to a universe of unforeseen antigens.

Instructional Theories

Instructional theories, based on the deterministic views of chemists such as Pauling (22), proposed that antigen determined the structure of the antibody. By using antigen as the direct template for antibody production, antibodies could be made to a vast number of antigens. This view, although unable to explain how self antigens were discriminated from foreign antigens, was widely held throughout the 1940's. During this time, Owen (23) reported that dizygotic twin cattle which exchanged blood *in utero* lacked the ability to make an immune response against the other's red blood cell antigens. Soon afterward, Burnet and Fenner (24) incorporated Owen's observation into a theoretical model which proposed a 'self-marking' process that occurred during an early stage of development. After a certain period of time, the immune system identified, as foreign, anything that lacked the self-marker. They postulated that antigen guided the formation of antibody by transmitting information to the genome, an 'indirect template' for the formation of specific antibodies. Accordingly, antigens introduced during development of the immune system would be regarded as self. Thus, this theory could account for self-nonself discrimination.

Based on Burnet and Fenner's theory (24), Billingham, Brent and Medawar (25) demonstrated acquired allograft tolerance. This group injected allogeneic lymphoid cells into neonatal mice. As adults, the inoculated animals were capable of accepting donor-strain skin grafts while rejecting third-party strain allografts. Coincidentally, Hasek (26) found that the parabiosis of chick embryos resulted in adult birds which were incapable of producing antibody in response to the parabiotic partner's red blood cells and were unable to reject skin grafts exchanged between them. These two experiments demonstrated that, as postulated by Burnet and Fenner (24), contact with foreign tissue very early in life resulted in its recognition as self.

Selection theories

Prior to 1955, chemists had a stronghold on immunological theory. At this time, a bold step was taken by Jerne (27) when he challenged the instructional theorists with a 'natural selection' theory for antibody formation. His theory was based on Darwinian ideas; adaptation resulted from the random generation of change and natural selection. Realizing that antibody to many antigens were present in serum of animals that had never been immunized, Jerne proposed that the host produced natural antibodies which bound to their specific antigen and transported it into cells. The antibody would signal the cell to reproduce molecules like itself and thus, large amounts of antibodies could be formed. Self-nonself discrimination was accounted for by a 'self-absorption' mechanism in which natural antibodies specific for self components were absorbed by tissues of the body and therefore could not mediate autoantibody formation.

Jerne's idea that the entry of an antigen-antibody complex into a cell stimulates replicas of the antibody seemed unlikely to Burnet (28) and Talmage (29). These investigators also held a Darwinian view that antigen selected preformed antibody. With the concept that nucleotide sequences of genes were responsible for protein structure, Talmage (29) proposed that antigen selected the cell and then the cell made copies of antibodies specific for that antigen. Burnet (28) hypothesized that a system that could respond to any number of unforeseen antigens required the random generation of receptor diversity, though the mechanism of generating this diverse repertoire was unknown. From these ideas, the clonal selection theory of antibody formation was postulated (30).

The clonal selection theory proposed that antibodies were natural products on cell surfaces. Antibody specificity was generated randomly and a specific antigen would signal the clonal proliferation of the particular cell, with each cell producing antibodies of one given specificity. Burnet realized that the random generation of the receptor repertoire and antigen selection of the appropriate cell left the potential for self-reactivity. He, therefore, proposed that *'forbidden clones'* capable of reacting with self antigens, were physically eliminated during ontogeny, a mechanism now referred to as *'clonal deletion'*. Clones destined to react with foreign antigens were allowed to mature. In addition to providing a model for self-nonself discrimination, the concept of clonal selection explained other experimental observations. The cellular division of one clone could account for the logarithmic, rather than arithmetic, rise in antibody titers. Also, the phenomenon of immunological memory, observed upon secondary exposure to antigen, could be explained by the increase in the number of antigen-specific clones which had been expanded following the first antigen exposure. Thus, the clonal selection theory could satisfactorily account for the nature of antibody responses and provide a basis for self-nonself discrimination.

A proposition of the clonal selection theory is that self antigen-specific clones are eliminated during ontogeny. Therefore, cells specific for the particular antigen should be absent from tolerant animals. Nossal and Pike (31) tested this idea with neonatal mice tolerized to the hapten fluorescein. They found that frequencies of hapten-specific B cells from these mice were similar to non-tolerant mice. However, when the specific B cells were stimulated with antigen in culture, their ability to produce antibody was impaired. Thus, antigen-specific B cells were physically present in tolerant animals but they lacked the ability to produce antibody in response to the antigen. Nossal coined the phrase *'clonal anergy'* to refer to this phenomenon.

The classical concept of alloreactivity: the transplantation paradox

The clonal selection theory, as with preceeding models, saw antigen as the sole inducer of the immune response. It followed that antigens of the major histocompatibility complex (MHC), initially defined by their ability to trigger allograft rejection, drove the allogeneic immune response. The classical view of allograft rejection saw these antigens as the major barrier to tissue transplantation; that is, allograft reactions were a direct response to MHC antigens that differed from the host. It was proposed that, since these antigens were genetically encoded, the only solution to the prevention of allograft rejection had to involve changing the host in a way that its response to the foreign antigen would be diminished (32), that is, either matching the MHC of the host to the graft or immunosuppressing the recipient. This has remained the predominant approach in experimental and clinical transplantation as tissue typing and host immunosuppression are based on the notion that transplantation antigens alone constitute the major barrier to allografting. There are, however, problems with this 'one signal' view of alloreactivity.

To Lafferty and Cunningham (33), this view of allogeneic reactivity was inadequate because it failed to explain several observations:

1). The species-specificity of graft versus host (GVH) reactions: When lymphoid cells from adult chickens were placed on the chorioallantoic membrane of immunologically immature allogeneic chick embryos, a graft versus host reaction ensued. The intensity of this GVH reaction decreased as the species disparity between donor and recipient increased. Despite larger antigenic differences, xenogeneic interactions were weaker than allogeneic reactions (34). This suggested that something more than antigen was required for activation of the allograft response. Similar observations made earlier by Simonsen (35) were attributed to "allergic death"; that is, the xenogeneic cells died in the face of overwhelming antigenic differences. However, Lafferty and Jones (34) demonstrated that lymphoid cells from pigeons could survive on the chorioallantoic membrane of the chick embryo and were capable of destroying pigeon bone engrafted on the membrane without reacting against the chick embryo. Since the xenogeneic cells were not dead, they concluded that a second signal, or costimulator (CoS), was required for immune induction and that this second signal displayed the species-specificity of GVH reactions.

2). Requirement for viable cells: In vitro analysis of allogeneic reactivity demonstrated that the activation of alloreactivity was a viable cell function of the stimulating cell population. Cells which expressed alloantigen but were heat-killed or metabolically inactivated by ultraviolet (UV)-irradiation could not stimulate in mixed leukocyte culture (MLC) (36-38).

3). Weak immunogenicity of isolated MHC antigens: Transplantation antigens, when removed from the cell surface, did not elicit strong immune responses (39-42).

These observations led to an important paradox in transplantation:

i) Allograft rejection is dependent on the recognition and response to MHC antigen carried on the graft.

ii) Allograft rejection is one of the most violent of immune reactions.

iii) MHC antigens, by themselves, are very weak immunogens (41, 42).

A solution to this transplantation paradox was provided by a two-signal model for T cell activation initially proposed by Lafferty and Cunningham in 1975 (33).

Two-signal models for lymphocyte induction: the Bretscher/ Cohn model

In 1970, Bretscher and Cohn (43) were satisfied with Burnet's proposal that diversity was established by random generation and mutation, and that each cell had a surface receptor with the same specificity as the antibody it produced (30). However, they were bothered by the idea that clones with anti-self reactivity were eliminated only during ontogeny, as mutations toward self-reactivity could theoretically also occur later in life. In addition, the clonal selection theory could not explain the finding that haptens could not immunize unless attached to a carrier molecule. To account for these phenomena, they introduced a theory of self-nonself discrimination in which two signals were required for lymphocyte induction (43). Signal one, antigen binding by the lymphocyte receptor, led to immune induction only in conjunction with a second signal simultaneously delivered by a carrier antibody. The recognition of foreign antigen by both receptors (linked associative recognition) was required for the simultaneous delivery of both signals. The hallmark of this theory was that signal one alone was tolerogenic. Thus, self antigens could be discriminated from foreign antigens throughout the life of the individual as any mutation resulting in a cell with anti-self specificity would deliver signal 1 only and lead to tolerance. For autoimmunity to occur, mutations to greater than one self determinant would have to occur simultaneously, an event that was assumed to be unlikely.

The two-signal model for T cell activation

Based on the Bretscher/Cohn model for lymphocyte induction, Lafferty and Cunningham proposed a two-signal model for allogeneic reactivity (33). The first signal was provided by engagement of the antigen receptor, and the second signal was a species-specific signal provided by a metabolically active stimulator cell. To maintain the self-nonself discriminatory capacity of the Bretscher/Cohn model, linked associative recognition of the antigen was absolutely necessary. As it was assumed that T cells had immunoglobulin-like receptors, Lafferty and Cunningham postulated that a cytophylic antibody on the stimulator cell was necessary to present antigen to the T cell. In this way, T cells received both signals simultaneously.

Around the time that Lafferty and Cunningham (33) were proposing this two-signal model for T cell induction, experiments by Zinkernagel and Doherty (44) demonstrated that T cells could 'see' antigen only in association with self MHC molecules. These experiments formed the basis for the current view of MHC restriction and, because of this view, the notion of the cytophylic antibody in the Lafferty/Cunningham model (33) was dropped. With it, the ability of the two-signal model for T cell activation to explain self-nonself discrimination had to be abandoned. Lafferty (45, 46) then modified the two-signal model for T cell activation to its present form by proposing:

1). Signal 1 is provided by engagement of the T cell receptor (TCR).

2) Signal 2 is the provision of a species-specific inductive molecule (CoS) by a metabolically active antigen-presenting cell (APC), designated as having an S^+ phenotype.

3). The production of CoS activity is regulated by the engagement of a control molecule 'c' on the surface of the APC.

Thus, this two-signal model for T cell activation provided an explanation for the species specificity of GVH reactions, the requirement for living stimulator cells in allogeneic reactions, and the weak immunogenicity of MHC antigens when they were removed from the cell surface. MHC antigens are strongly immunogenic only when they function as the control molecule on the surface of an active APC. The inference from this model, then, is that MHC antigen alone is not immunogenic.

Experimental support for the two-signal model

Experimental evidence supporting the two-signal model for T cell activation comes from observations made on the ability of tumor cell lines to stimulate allogeneic T cells *in vitro* (47).Gamma-irradiated P815 (H-2^d) tumor cells presented Class I MHC antigen and stimulated a strong cytotoxic T lymphocyte (CTL) killing response. Such tumor cells, on the basis of providing both signals necessary for T cell activation, expressed a stimulator (S^+) phenotype. On the other hand, γ-irradiated CaD2 (H-2^d) tumor cells, or P815 cells which had been UV-irradiated to inhibit their metabolic activity, could not elicit a CTL response. These cells were of a nonstimulatory, S^- phenotype. The addition of exogenous cytokines, in the form of Concanavalin A (Con A) stimulated spleen cell supernatants (SN), reconstituted the CTL response when added to the S^- cell-bearing cultures (46). This indicated that the S^- cells expressed recognizable alloantigens, providing evidence that alloantigen, by itself, was not sufficient to activate the *in vitro* response.

The above experiment also provides support for the nature of the CoS as a soluble, inductive molecule. In contrast to this notion, Sprent and Schaefer (48) proposed that the S^+ phenotype of the APC comes from quantitative cell surface properties such as antigen density or accessory molecules on the cell surface. More recently, the B7/BB1 accessory molecule expression on APCs and activated B cells (49) is thought to play a CoS function via its interaction with CD28 (50) or CTLA-4 (51) molecules on T cells. A recent report has indicated that the cytoplasmic domain of the Class II MHC molecule is required to transmit signals necessary for B7 expression (52). This evidence supports the role for MHC molecules as the 'control' molecules regulating CoS activity, an implication of the two-signal model (46). In addition to B7, accessory molecules such as ICAM-1 and LFA-3 may also have costimulatory properties (53). Thus, the exact nature of costimulatory signals required for T cell activation still needs to be resolved.

Implications of the two-signal hypothesis: the stimulator cell model

The theory of allogeneic reactivity (Appendix A), based on the two-signal model for T cell activation, implies that antigen can be presented in either an active or a passive manner. **Active** antigen presentation occurs when MHC antigen is presented to specific T cells on the surface of metabolically active stimulator (S^+) cells, that is, cells capable of supplying CoS activity required for T cell activation. **Passive** antigen presentation occurs when MHC antigen is presented on the surface of cells, such as tissue parenchymal cells, which are incapable of supplying the second (CoS) signal (S^- cells). This indicates that transplanted tissue will be a mosaic of S^+ and S^- cells, that is, cells which can deliver CoS activity and activate T cells and parenchymal cells which express MHC antigen but cannot provide the second signal. Therefore, allograft immunogenicity will be dependent on the presence of S^+ cells. The key implication of this theory is that removal of active APCs from an allograft prior to transplantation would prevent the activation of graft-specific immunity and facilitate allograft acceptance.

Implication of the stimulator cell model: modification of tissue immunogenicity

In contrast to the classical concept of allogeneic reactivity (32), the stimulator cell model implies that allograft rejection could be prevented by treating the tissue rather than the recipient, an idea proposed in the early 1930's when pre-transplant culture of human parathyroid tissue was shown to be beneficial (54). The concept at the time was that the graft could adapt to the host if it was bathed in the recipient's serum. Without a sound theoretical basis, these observations were disregarded. In 1957, Snell (39) proposed a passenger leukocyte model which suggested that leukocytes residing in donor tissue provided the major stimulus for allograft rejection due to their ability to migrate to local lymphoid organs. Ten years later, Steinmuller (55) demonstrated that graft immunogenicity was due to a population of hematopoietic cells carried within the graft. A few years later, a report by Summerlin *et al* (56) indicated that organ culture facilitated allogeneic skin graft survival although this was not confirmed. This report prompted Lafferty *et al* (57), with the backing of the stimulator cell model, to test the prediction that the removal of APCs from donor tissue would result in prolonged allograft survival. They reported that short-term culture of thyroid tissue in an atmosphere of 95% O_2 and 5% CO_2 extended graft survival in untreated, allogeneic recipients. Extending the time in culture led to indefinite thyroid allograft survival (>100 days) (58). Further studies demonstrated that cyclophosphamide pretreatment of the tissue donor could reduce the period of high O_2 culture necessary to achieve indefinite thyroid allograft survival to 7 days (59). Increasing pressure to 2.5 atmospheres for 48h (60) or culturing in an acidic (pH 5.5) salt solution for 24h (61) also resulted in indefinite allograft survival. Though high O_2 culture was selected serendipitously, leukocytes were later found to be extremely sensitive to high O_2 tensions (60) and high O_2 organ culture led to the degeneration of the vascular bed and hematopoietic cells within cultured tissue, leaving only tissue parenchymal cells (62). Taken together, this evidence supports the role of S^+ cells in triggering allograft rejection. These findings demonstrate that pretreatment of the transplanted tissue results in indefinite allograft survival without a need for recipient immunosuppression.

These initial studies on thyroid allografts have been extended to a variety of small endocrine tissues including ovarian tissue (63), parathyroid (64), keratinocytes (65) and pancreatic islets (66-68) in both mouse and rat models. Because little or no immunosuppression is necessary, and because immunosuppressive techniques would not be compatible with clinical islet transplantation, many of the attempts to prevent islet allograft rejection utilize this approach of modifying tissue immunogenicity prior to transplantation. In addition to high O_2 culture, several other pre-transplant regimens have been reported. Based on reports that stimulator cells cultured at 22°C (69) or exposed to UV-irradiation (37) failed to activate allogeneic cells in an MLC, low-temperature culture (70) or UV-irradiation techniques (71) were used to modulate rodent islet immunogenicity. In most cases, indefinite (>100 days) allograft survival was obtained provided a short course of immunosuppression, either anti-lymphocyte serum (ALS) (66), or cyclosporin A (CsA) (72, 73), was given at the time of transplantation. Without immunosuppression, indefinite murine islet allograft acceptance has been obtained by pretreating islets with donor-specific Ia antisera (74, 75) or a monoclonal anti-dendritic cell antibody (76) and complement. An *in vitro* nonenzymic procedure for the isolation of islets from fetal or perinatal rats (77) leaves islets devoid of Class II$^+$ cells (78, 79) and results in indefinite islet allograft survival, though graft survival is strain dependent (80, 81). These latter studies were based on a notion that Class II MHC antigen expression was a marker for cells expressing the S$^+$ phenotype. Although a correlation exists between Class II-positivity and the S$^+$ phenotype, this is not always the case (82, 83). The ability to modulate tissue immunogenicity by a variety of procedures provides strong support for the stimulator cell concept. The significance of these observations is that MHC antigen carried on the parenchymal cells of transplanted tissue is not sufficient to activate an alloimmune response.

Antigenicity versus immunogenicity of MHC alloantigens

The prevailing view of alloreactivity prior to the 1970's was that transplantation antigens, *per se*, were the stimulus for allograft immunity: that is, graft antigenicity plays the dominant role in allograft rejection. Thus, a major concern with the attempts to modulate tissue immunogenicity has been whether pretreated tissues remain antigenic and, thus, capable of being recognized. Is the acceptance of pretreated allografts due to the elimination/inactivation of stimulator cells within the tissue or to a modification of graft antigens? An early report by Jacobs and Huseby (84) attributed the survival and growth of organ cultured tumors in allogeneic recipients to the alteration of graft antigens. Recently, several methods of tissue pretreatment; culture in hyperbaric oxygen (95% O_2, 25 psi, 48h) (85, 86), at low pH (86) or at 24°C (87), have been reported to decrease cell surface MHC antigen expression. The implication made in two of these studies was that, in addition to APC depletion, decreased graft antigenicity was also necessary for prolonged graft survival (85, 87). Hullett *et al* (85) reported that, following hyperbaric oxygen culture, Class I MHC antigen expression on thyroid tissue was undetectable by immunoperoxidase staining, even after a 48h incubation in interferon-gamma (IFN-γ). Such cultured allografts survived in donor skin graft primed recipients and could resist rejection by *in vitro* primed donor-specific cytotoxic T cells. These findings indicated that graft antigenicity played a role in allograft survival and this group concluded that the loss of APC function may not be the primary reason for the survival of pretreated tissue. Thus, the role of the APC in allograft immunity remains controversial.

Several lines of evidence indicate that, although MHC antigen expression mediates the efficient activation of allogeneic T cells, as indicated by the survival of Class I MHC deficient islets in allogeneic recipients (88), it is not *sufficient* for allograft rejection:

1). Batchelor *et al* (89) investigated the mechanism of long-term kidney allograft survival in immunologically enhanced recipients. When long-surviving kidneys were regrafted into untreated secondary recipients, they did not elicit an immune response. This failure of retransplanted kidneys to activate an immune response was not due to a modulation of Class I or Class II MHC antigen expression (89, 90). In fact, host immunization with very small numbers of donor strain dendritic cells (a source of S^+ cells) led to rejection of the established kidney graft (91). Thus, alloantigens on the grafted kidney did not induce an immune response but could serve as targets for destruction following host immunization.

2). Although the induction of Class I and Class II MHC antigen has been shown to occur during rejection of allografts in many experimental and clinical situations (92-94), it is not necessarily indicative of graft rejection. For example, following donor-specific blood transfusion (DSBT), Class I and Class II MHC antigen expression is increased on rat renal allografts whether the grafts are rejected or tolerated (95). In addition, the intentional upregulation of MHC antigen expression on thyroid or islet tissue by pretreatment with IFN-γ, does not increase the susceptibility of allografts to rejection (96, 97). La Rosa and Talmage (86) demonstrated that when high O_2 cultured thyroids were incubated in recombinant IFN-γ to increase MHC antigen expression prior to transplantation, these grafts did not become susceptible to rejection. They functioned in non-immunosuppressed hosts as well as control cultured allografts in which MHC antigen expression was not upregulated. Thus, increasing antigenicity did not result in increased immunogenicity.

3). The advent of transgenic technology allowed examination of the issue of antigenicity versus immunogenicity of Class II MHC antigens. Markmann *et al* (98) used transgenic mice in which MHC Class II I-E molecules were exclusively expressed on the surface of pancreatic islet β cells in I-E^- mice (99). I-E^+ fetal pancreata, from such transgenic mice, were implanted into naive (I-E$^-$) recipient mice such that I-E was present on islets but not on APCs. Although these grafts did not elicit an alloimmune response, they were promptly rejected when the recipient was immunized with I-E$^+$ spleen cells. This experiment demonstrated that Class II MHC antigen, *per se*, is not sufficient to trigger allograft immunity efficiently.

4). APC-depleted allografts demonstrate the distinction between graft *antigenicity* and graft *immunogenicity*. Although they do not elicit an immune response *in vivo*, such tissue is still antigenic. The evidence for this came from demonstrations that established cultured allografts were acutely rejected when hosts were immunized with donor-strain peritoneal exudate cells as a source of APCs (58, 100). These studies and others (101, 102) indicated that cultured allografts retained recognizable cell surface antigen. Therefore, an alteration of graft antigen was not necessary for a reduction of tissue immunogenicity.

Thus, Class I or Class II MHC alloantigens, *per se*, are not inherently immunogenic and are not the major barrier to successful allografting as suggested by the classical model of allograft rejection. Overall, these studies indicate that, although antigenic modulation may occur as a consequence of some forms of immunomodulation, a loss of antigen is not required for a loss of immunogenicity. Likewise, increased alloantigenicity should not be equated with increased immunogenicity. Thus, MHC antigen expression is required but not sufficient for allograft rejection.

ALLOGRAFT TOLERANCE

The rationale behind attempts to induce tolerance to tissue allografts is to obviate the need for immunosuppressive agents due to the deleterious effects associated with their use. It will be essential to determine how tolerance is induced and how the tolerant state is maintained so that: 1) the tolerant state can be detected in patients currently on immunosuppressive drugs and 2) future strategies can be directed towards inducing a tolerant state.

Since its initial description by Billingham, Brent and Medawar (25), the literature has been inundated with empirical approaches resulting in allograft tolerance. Approaches to the induction of donor-specific allograft tolerance are varied, reflecting the fact that tolerance can be induced in many ways in both neonatal and adult animals. Some of these approaches manipulate a newly developing immune system, either a neonatal environment (103) or an adult immune system that has been completely ablated such that it resembles the neonatal state. The latter would include irradiation chimeras (104, 105) or intrathymic grafting with ALS (106). Other methods utilize a host transiently immunocompromised by agents such as immunosuppressive drugs (107-110) or monoclonal antibodies (mAb) (111). A third approach, utilizing immunocompetent recipients, includes pretreatment of the recipient with anti-donor antibody (112) or donor antigens (113, 114), commonly in the form of donor-specific blood transfusions (115). In addition, pretreatment of the donor tissue to eliminate immunogenicity can eventually lead to tolerance induction (116-118). Many models combine methods such as: 1) bone marrow grafting with ALS (119, 120) or mAb (121), and 2) anti-T cell mAb in combination with irradiation (122) or donor antigen (123). These studies illustrate the many ways in which tolerance can be induced. However, despite much effort, the mechanisms responsible for the tolerant state are still speculative. The development of a theoretical framework within which these observations can be incorporated, and from which testable hypotheses can be made, would aid in future clinical application.

Definitions of Tolerance

Because immunological tolerance is such a diverse area of investigation, confusion often arises from the lack of a clear definition of the tolerant state. For example, the long-term acceptance of an allograft in the absence of continuous immunosuppression is sometimes equated with tolerance. The distinction between graft acceptance and tolerance induction can be illustrated by the acceptance of an APC-depleted graft or a graft placed in a privileged site. In such instances, grafts can survive without host tolerance induction (124). Therefore, transplantation (allograft) tolerance will be defined as: *a state of specific altered reactivity that allows the acceptance of a graft that would otherwise be rejected* (125). In addition, the following forms of tolerance require clear definitions.

Clonal Deletion

The theories of self-nonself discrimination by Burnet (30) and Bretscher and Cohn (43) proposed that the self-reactive cell was physically eliminated. This clonal deletion of self-reactive cells is the most unambiguous form of tolerance. In recent years, deletion has been shown to be a major mechanism of tolerance to self components in the thymus (126-128). It may also occur in the periphery as a result of massive clonal expansion and 'exhaustion' (129, 130) or via a veto mechanism (131).

Clonal Anergy/Inactivation

The term 'clonal anergy' has been revitalized by Schwartz and co-workers (132) following their demonstration that antigen presented by chemically fixed APCs led to the inactivation of specific T cell clones (133). Such inappropriate antigen presentation (signal 1 without provision of the second CoS signal) was referred to as T cell clonal anergy. In recent years, experimental findings are often interpreted in terms of 'clonal anergy'. Lacking a common definition, 'anergy' has become a global term encompassing situations such as: 1) the inappropriate presentation of antigen (signal 1 only) as described by Schwartz (132), 2) receptor desensitization due to repeated stimulation (134), 3) down-regulation of TCR and/or co-receptors (135), and 4) lack of T cell activation or 'help' (98, 136, 137). Often, any lack of *in vivo* reactivity, whether or not *in vitro* reactivity occurs, is referred to as anergy. For clarity, clonal anergy will be defined as a state in which antigen exposure renders cells bearing appropriate receptors refractory to subsequent immunogenic stimuli. Thus, both deletion and anergy are passive mechanisms, *intrinsic* properties of the antigen-specific T cell.

Suppression/Regulation

In the early 1970's, Gershon and Kondo (138) and McCullagh (139) published results of experiments in which cells from tolerant animals were capable of suppressing the ability of normal cells to react to the specific (tolerated) antigen. That is, a mixture of tolerant lymphocytes and normal lymphocytes responded as if tolerant. At the time, all tolerance was thought to be due to the deletion of antigen-specific clones, in which case, mixtures of tolerant and normal lymphocytes would be expected to be reactive to the antigen. Instead, tolerance was dominant. Several examples of suppressive forms of tolerance have been demonstrated in allograft transplantation models (118, 140, 141). Thus, active suppressive (regulatory) mechanisms will include states in which antigen-reactive T cells are negatively regulated by cellular or humoral factors. As such, suppressive tolerance will refer to factors *extrinsic* to the reactive T cell.

INDUCTION OF TOLERANCE

There are at least three important issues in considering the induction of the tolerant state: 1) the relative contributions of central (thymic) versus peripheral environments, 2) the timing of induction relative to the maturation of the T cell and 3) the form of antigen presentation, that is, which signals must be presented to induce the tolerant state.

Site of tolerance induction: thymus versus periphery

Thymic Environment

The role of the thymus in T cell development has been realized for some time (142). The general view of its function is that it must: 1) delete autoreactive T cells to maintain self-tolerance (*negative selection*) and 2) nurture T cells that will recognize foreign antigen in association with self MHC in the periphery (*positive selection*), a process necessary to explain the phenomenon of MHC-restriction (44). How the thymus accomplishes this task is a complex issue. A simplistic view of this process is that precursor T cells enter the thymus, acquire cell surface CD4, CD8 and TCR molecules and then meet with one of three fates depending on the specificity of the TCR (143-147). If the TCR fails to recognize any component in the thymus, the cell dies. Double-positive ($CD4^{+}CD8^{+}$) thymocytes bearing TCRs that bind to self MHC antigens expressed on bone-marrow derived thymic APCs are deleted (negatively selected). Thymocytes bearing TCRs that bind MHC antigen on thymic epithelium are rescued from cell death and allowed to mature (positively selected). Since both selection events involve T cells having some affinity for self MHC molecules, it has been proposed that those cells with relatively high affinity for self MHC are deleted and those with relatively low affinity for self MHC antigen, are positively selected. The differentiation of $CD4^+CD8^+$ cells to single positive $CD4^+CD8^-$ or $CD4^-CD8^+$ mature T cells is based on the MHC specificity of the TCR; $CD8^+$ T cells are restricted to Class I MHC antigens and $CD4^+$ T cells are MHC Class II-restricted. The nature of the signals received by the thymocyte, its maturation stage and the intrathymic site of these events are controversial issues.

Until recently, intrathymic events were difficult to decipher; due to low precursor frequencies, the fate of a given T cell could not be followed. Direct evidence for both negative and positive selection events has now been obtained in systems utilizing either superantigens (SAg) (147) or TCR transgenic mice (148). Both models have contributed much to the understanding of intrathymic selection events because large numbers of T cells with a given marker can be identified with mAb. Superantigen models analyze TCR V_β families that share variable region determinants on the β chain. Monoclonal antibodies bind the TCR of known V_β families and can detect a large heterogeneous population of T cells in unmanipulated mice (147, 149, 150). The first direct evidence for deletion occurring in the thymus came from the Kappler and Marrack group (126) who demonstrated that immature thymocytes bearing $V_\beta 17_a{}^+$ TCRs were present in mouse strains bearing MHC Class II I-E antigen. However, mature T cells expressing these TCRs were absent in the periphery of these mice (126), indicating that the precursors were deleted. This deletion of T cells bearing I-E specific TCR V_β domains has been shown to be due to the co-recognition of endogenous mouse mammary tumor viral SAg (151, 152). Thus, T cell recognition is not for the MHC molecule, *per se*. Similar results have been demonstrated for TCR V_β families responsive to other SAg determinants, such as Mls (147, 149, 153). In addition, nondeletional (anergy) mechanisms of intrathymic tolerance have been reported using SAg models (147, 154-156).

The advantage of these models is that SAg stimulate T cells bearing a given V_β family and this corresponds to a large proportion of total T cells. However, because the combining site of the TCR is molded by both α and β chains, and because SAg bind MHC outside the conventional peptide groove (157), these models do not represent conventional TCR-MHC+antigen interactions. This problem was overcome when von Boehmer's group developed TCR transgenic mice in which the majority of T cells had receptors of the same specificity (127). The advantage of this approach was that high frequencies of a single T cell receptor with specificity for a known conventional antigen plus MHC could be followed. This group utilized TCR transgenic mice bearing T cells specific for the male H-Y antigen in the context of H-2D^b MHC antigen (127). Such T cells were identified in the thymus of both female and male transgenic mice, but would only seed the periphery of female mice, indicating that the self (H-Y)-reactive thymocytes were deleted in the H-Y^+ male animals. Similarly, evidence for positive selection came from TCR transgenic mice (158-160). For example, transgenic T cells restricted to H-2^b could mature in syngeneic (H-2^b), but not allogeneic (H-2^d), mice. Thus, T cells matured only when the appropriate restricting MHC haplotype was present. In this way, TCR transgenic, as well as SAg, models have been useful in demonstrating the role of the thymus in self-nonself discrimination.

This type of evidence for negative and positive selection events demonstrates that the thymus plays a major role in the deletion of autoreactive clones but it also raises questions as to whether clonal deletion is sufficient to account for all self tolerance. Deletion of self-reactive clones occurs when thymocytes see self antigen on thymic APCs but it is unlikely that all self antigens are present in the thymus. Also, it appears that self-reactive T cells and B cells are present in normal individuals. Polyclonal activation of B cells with mitogens induces antibodies reactive to self components (161,162). In addition, experimental models of autoimmune diseases can be induced by appropriate immunization with self antigens. For example, experimental allergic encephalomyelitis, a model of multiple sclerosis, can be induced by injection of myelin basic protein in complete Freund's adjuvant (163). Likewise, injection of acetylcholine receptor protein induces experimental myasthenia gravis (164) and experimental models of rheumatoid arthritis can be induced with collagen and/or adjuvant alone (165). These studies indicate that T cells specific for self antigens exist in the periphery of normal animals and can be activated under appropriate conditions.

Peripheral Environment

How, then, does the immune system deal with self-reactive T cells that escape negative selection in the thymus or that bear TCRs specific for antigens that are uniquely extrathymic? The encounter of such T cells with peripheral self antigen may potentially activate the T cell, tolerize the T cell or neither activate nor tolerize the T cell. Many investigators believe that mechanisms of tolerance must be imposed in the peripheral (extrathymic) environment to prevent activation of autoimmune responses. This issue has been addressed in SAg models or in transgenic studies in which foreign antigens are introduced via a transgene, regulated by a tissue-specific promoter. In most cases, functional tolerance develops to the transgene, either Class I (166, 167) or Class II (168) MHC alloantigens or viral antigens (169). Though deletional forms of tolerance have been proposed to occur in the periphery (129, 130), nondeletional mechanisms, including clonal anergy (99, 170) or down-regulation of the TCR and/or co-receptors (135) have been reported most often. In most of these models of tolerance to extrathymic antigen, *in vitro* responses to the antigen of interest were diminished relative to nontransgenic controls (99, 166, 171). Several models postulating T cell anergy induction have demonstrated strong T cell proliferation in response to stimulation *in vitro* with hyporeactivity *in vivo* (136, 137, 172, 173). In contrast to these studies, Ohashi *et al* (174), utilizing a viral transgenic mouse model, suggested that extrathymic antigen was essentially ignored by mature T cells. Thus, the contributions of the thymus versus the periphery in self-nonself discrimination remains a controversial issue.

Stage of lymphocyte maturation

The stage of lymphocyte maturation is related to the site of tolerance induction in that immature T cells are found predominantly in the thymus. Burnet (30) postulated that the process of self-nonself discrimination during ontogeny of the immune system determines what lymphocytes will regard as 'self' or as 'nonself'. In a transgenic model of peripheral tolerance in which the SV40 T antigen was expressed exclusively on pancreatic islets, mice were tolerant of the antigen if it was expressed early during ontogeny (175). However, if the transgenic product was expressed later in development, autoimmunity ensued. Thus, once the self-nonself decision has been made, foreign 'nonself' antigen can trigger T cell activation.

There has been a persistent idea that immature T cells are more susceptible to tolerance induction than are mature T cells. Whether this is due to an intrinsic property of the immature thymocyte itself or to specialized properties of the thymic environment is not clear. Matzinger and Guerder (176) demonstrated that splenic APCs, which led to the activation of mature T cells, were also capable of inactivating immature thymocytes. Similarly, Swat *et al* (177) demonstrated that the TCR-MHC interaction which induced deletion of immature T cells, induced the proliferation of mature T cells. It has also been shown that immature and mature T cells have different avidity thresholds for signal transmission: low avidity interactions which cause deletion of immature thymocytes are not sufficient for the activation of mature cells (178, 179). These studies suggest that an intrinsic property of the immature T cell may make it more susceptible to tolerance induction than mature T cells.

The extent to which new thymic emigrants are susceptible to tolerance induction is debatable. Sprent (180) has proposed that fully mature T cells are resistant to tolerance induction, although deletion of mature autoreactive T cells has been observed (129, 130). When mature H-Y reactive T cells from TCR transgenic mice were injected into athymic male *nu/nu* (nude) mice, they proliferated vigorously and then were deleted from the periphery (129). The few remaining H-Y specific T cells were rendered anergic in that they did not respond *in vitro*. Similar events occurred when Mls^a cells were injected into Mls^b mice (130). Both situations involve large numbers of T cells which encounter very large doses of antigen in the periphery. Thus, mature T cells *can* be susceptible to antigen-dependent elimination. In allograft tolerance, the greater susceptibility of immature cells to tolerance induction would explain why tolerance to allografts is readily induced in the neonate. However, since allograft tolerance in adult animals involves mature T cells, the goal of transplantation will be towards manipulating the immunocompetent cell.

Nature of antigen presentation

The Lafferty/Cunningham (33, 46) two-signal model for T cell activation, derived from the Bretscher/Cohn (43) model for lymphocyte induction, proposed that two distinct signals: 1) engagement of the TCR and 2) CoS activity provided by a metabolically active (S^+) APC were necessary for induction of an immune response. The signals required for the induction of tolerance have not been established. A proposition of both the Bretscher/Cohn (43) and Lafferty/Cunningham (33) models has been that signal 1 alone was tolerogenic; that is, antigen alone delivered a negative signal to the reactive cell. This issue is controversial as there is evidence for both one and two signal requirements for tolerance induction.

Signal one alone induces tolerance

The notion that signal one alone was tolerogenic was renewed by Schwartz and co-workers who provided *in vitro* evidence that inactivation of specific T cell clones occurred when antigen was presented by chemically-fixed APCs (signal 1 delivery) (133). Such T cell clonal anergy also results when antigen is presented by planar cell membranes, or when the TCR is stimulated by immobilized anti-CD3 antibody or Con A in the absence of APCs (132). This anergic state is characterized by a decrease in proliferation following a subsequent immunogenic stimulus.

The induction phase requires protein synthesis and a rise in intracellular Ca^{++} (132). Though the T cells increased in size, they made reduced amounts of IL-3/GM-CSF and IFN-γ, showed reduced expression of IL-2 receptors, and were unable to produce their own growth factor (IL-2) (181, 182). Significantly, this anergic state could be rescued by bystander APCs, indicating that the second signal could be delivered independently of the antigen-specific first signal (183). Similarly, stimulation of anergic cells with exogenous IL-2 completely reversed this state (182). Once induced, the anergic state lasted for several weeks but, over time, it diminished in the absence of TCR occupancy. Overall, this *in vitro* evidence suggests that occupancy of the TCR (signal 1) in the absence of a second CoS signal can lead to the inactivation of T cells such that they are refractory to a subsequent immunogenic stimulus.

Although designed to investigate peripheral tolerance, several transgenic models also address the issue of 'signal 1' antigen presentation *in vivo*. In these models, antigen is expressed exclusively on selected peripheral parenchymal cells which lack the capacity to generate CoS signals (S^- cells), not on hematopoietic S^+ cells. Thus, it was assumed that the thymus would play a neutral role in tolerance induction. When the rat insulin promoter (RIP) was used to direct expression of the MHC Class I K^b (170, 171) or Class II I-E (99) antigen to islet β cells, thymic expression of the transgene was not detected by immunohistochemical techniques. Islet inflammation, indicative of an autoimmune response, was not observed in these transgenic mice (99, 171, 184) even when mice were immunized with spleen cells bearing the relevant alloantigen (171). In addition, RIP-K^b transgenic mice maintained K^{b+} skin grafts yet were able to reject third-party strain skin grafts, indicating that these animals were tolerant to the transgenic product. *In vitro* CTL responses were specifically reduced in the RIP-K^b transgenic mice but could be overcome by recombinant IL-2 (171). Lymphoid cells from Class II transgenic mice could not be stimulated with mAb specific for the putatively Class II-reactive V_β TCRs and were unable to respond to IL-2 (168). Both groups of investigators concluded that anergy was induced by signal 1 antigen presentation in the absence of CoS signals.

Although these findings appear to support the notion that antigen presentation, in the absence of the second CoS signal, leads to clonal anergy, there are problems associated with these studies. First, MHC Class II I-E dependent tolerance is not due to allo-MHC recognition *per se*. T cells expressing certain TCR V_β families display enhanced reactivity to I-E. There is evidence to suggest that, by itself, the I-E gene product is not sufficient to mediate intrathymic deletion of such I-E reactive T cells (185). Rather, deletion of such T cells has been shown to be mediated by retrovirus-encoded SAg (151, 152). Thus, because of this SAg association, findings with the Class II I-E transgenic, like other models of anergy utilizing SAg (154, 186), may not reflect conventional antigen presentation.

A second problem with transgenic studies is the potential for the expression of the transgene product within the thymus at levels undetectable by immunohistochemistry. Although thymic expression of the Class I K^b transgenic product was not detected by immunohistochemical techniques, Northern blotting or S1 nuclease mapping, it *was* detected by amplification with the polymerase chain reaction (166). In fact, thymus grafts from RIP-K^b transgenic mice were able to induce tolerance to K^{b+} skin grafts in nontransgenic animals (187). This study and others (188) indicate that the nondeletional tolerance induced in these models may be due, in part, to the low level expression of the transgene product in the thymus and, thus, are not models of extrathymic (peripheral) tolerance at all. The findings that T cells from some transgenic models do not respond to stimulation *in vitro* could be accounted for by the deletion of high affinity T cells, leaving cells of lower affinity which cannot respond *in vitro* unless supplemented with IL-2. These studies point to the difficulty of ensuring exclusive extrathymic expression in transgenic mouse models.

T cell indifference to peripheral self antigen

It is not clear whether the lack of T cell autoreactivity in transgenic models is due to an antigen-specific event that leads to tolerance induction or due to an inability of the T cell to be appropriately activated. In contrast to the notion that signal 1 alone tolerizes, is the idea that signal 1 antigen presentation is a null event, that this form of antigen presentation neither activates nor tolerizes the specific T cell. Support for this concept came from Ohashi *et al* (174) who directed the expression of a lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) transgene to pancreatic β cells via the RIP (RIP-GP). Autoimmunity, as assessed by insulitis and diabetes, or tolerance assessed by a lack of *in vitro* CTL activity, was not observed in these RIP-GP transgenic mice. Even when the number of T cells specific for the GP antigen was increased by crossing RIP-GP mice with GP-specific TCR transgenic mice, neither T cell immunity nor tolerance was observed. Only when these animals were primed with live LCMV, did they develop diabetes demonstrating that an immune reaction could take place under appropriate conditions. This study indicated that peripheral T cells, encountering the foreign antigen on cells incapable of supplying the second signal, were neither activated nor tolerized.

Two other model systems have obtained parallel results. One of these systems utilized MHC Class II I-E transgenic mice in which I-E was targeted to pancreatic β cells (99). Markmann *et al* (98) used these mice as donors for I-E$^+$ fetal pancreas grafting into I-E$^-$ recipients. In this way, the I-E gene product was expressed only on S$^-$ islet β cells, not on hematopoietic S$^+$ cells and not in the thymus. The results showed that these I-E$^+$ grafts were accepted in I-E$^-$ recipients. However, when recipients were immunized with I-E$^+$ spleen cells, as a source of S$^+$ cells, the grafts were rejected. Therefore, prior to immunization, the host neither rejected nor became tolerant of the graft. Similarly, high O_2 cultured, APC-depleted allografts residing in an immunocompetent animal are not rejected unless the host is immunized with donor strain spleen cells in the early post-transplant period (46). Like the experiment of Ohashi *et al* (174), these two lines of evidence indicate that antigen presentation on the surface of cells which cannot supply the second CoS signal, does not appear to induce a tolerogenic signal which leads to clonal deletion/anergy of antigen-reactive T cells.

Tolerance and tissue immunogenicity

The studies cited above suggest that antigen presentation by signal one alone could be either a tolerizing event or a null event. Other studies suggests that two signals, while necessary for T cell activation, may also be required for tolerance induction to self components and to allografts. Cyclosporin A was used in the following studies because it blocks cytokine production and, therefore, may inhibit CoS events which are cytokine-dependent (189, 190). CsA has been shown to inhibit the progression of immature thymocytes to mature T cells and prevent the clonal deletion of self-reactive thymocytes (191). The prevention of clonal deletion by CsA may help explain the paradoxical effect this immunosuppressive drug has in inducing autoimmunity when irradiated rodents (192) or humans (193), which have received autologous bone marrow transplants, are withdrawn from the drug. In a similar manner, administration of CsA to neonates can induce autoimmune disease (194). The sensitivity of clonal deletion to CsA in these studies suggests that self tolerance induction may be a two-signal event.

Other evidence indicates that allograft tolerance induction, in adult animals, may be a function of tissue immunogenicity, that is, requiring 'two signal' presentation. Monaco's group (195) has demonstrated that tolerance to allogeneic islets in ALS-treated recipients is induced more efficiently when impure, immunogenic preparations are used rather than highly purified islets. This study indicates that tissue immunogenicity may facilitate tolerance induction. A more direct demonstration of this comes from a study in which CsA-treated rats were grafted with allogeneic islets which were either untreated or depleted of APCs by a period of high O_2 culture (196). CsA-induced tolerance, defined by the ability of the host to resist graft rejection with primed donor-reactive T cells, was observed only when untreated (S^+) tissue was grafted, not when cultured (S^-) tissue was grafted. This indicated that CsA-induced tolerance required immunogenic signals from the graft. These examples suggest that two signals may be necessary for tolerance induction. Thus, the nature of the signals required for tolerance induction needs to be clarified.

MAINTENANCE OF TOLERANCE

The studies described above indicate that several factors influence the induction of the tolerant state. Once induced, the tolerant state may be maintained by passive or active mechanisms. Passive mechanisms (deletion/inactivation) would manifest as a *recessive* form of tolerance, that is, a state which can be 'broken' by injections of normal cells, syngeneic to the recipient. Active regulatory mechanisms would be *dominant* over normal non-tolerant cells.

Passive Mechanisms of Tolerance

Clonal Deletion

Despite the numerous publications of passive mechanisms maintaining central or peripheral tolerance to self antigens (146, 166), there are relatively few reports of allograft tolerance induced in adult animals maintained by the deletion/ inactivation of donor-reactive T cells. Clonal deletion has been observed when allograft tolerance is induced in neonatal (103) or bone marrow chimeric (105, 197, 198) animals. In these situations, lymphoid cells from tolerant animals do not respond to donor alloantigens *in vitro*. The tolerant state is associated with decreased donor-specific CTL precursor (CTLp) frequencies and with stable chimerism in the thymus and peripheral lymphoid organs. Tolerance in these models could be abolished in these recipients by the infusion of naive syngeneic T cells, illustrating that a passive form of tolerance is recessive (103, 199). It should be noted that not all models of tolerance induced in neonatal animals or irradiation chimeras are maintained by deletion; active mechanisms have also been implicated (103, 200, 201).

Deletion of donor-reactive T cells may also occur via a veto mechanism. The veto phenomenon, originally described by Miller (202), is a form of tolerance in which a cell of hematopoietic origin (the veto cell) kills any cell that specifically recognizes and interacts with its MHC. Whenever a CTLp is generated, a veto cell can eliminate it. This mechanism is thought to eliminate self MHC-reactive T cells (131) but has also been proposed as a mechanism of allograft tolerance (203). Deletion in models of allograft tolerance requires the presence of donor hematopoietic cells during T cell development and stable chimerism. Hematopoietic chimerism may provide a continuous source of donor-derived veto cells necessary for the maintenance of the tolerant state and has been observed in patients years after successful allograft transplantation (204, 205).

Clonal Inactivation/Anergy

There is little evidence to date suggesting that allograft tolerance is due to the inactivation of donor-reactive T cells. It is also noteworthy that a clonal inactivation mechanism has not been reported when tolerance is induced in completely MHC-disparate strain combinations. The inactivation of I-E reactive T cells has been reported when pancreatic islets from MHC Class II I-E$^+$ mice were grafted to anti-CD4 treated I-E$^-$ recipients (206). Relative to unmanipulated I-E$^-$ mice, *in vitro* T cell responses to the appropriate Vβ family-specific mAb were reduced in tolerant mice. Similarly, when tolerance to Mls-disparate bone marrow grafts was induced by anti-CD4 and anti-CD8 mAb therapy, Mls-reactive T cells from tolerant animals responded poorly to *in vitro* stimulation (207). It is important to note that the proposed anergy of alloreactive T cells is *inferred* from the inactivation of Mls (SAg)-reactive T cells which may not be a typical model of antigen-specific interactions. In addition, in the latter study (207), the tolerant state could *not* be broken by the infusion of naive, syngeneic spleen cells. This finding indicates that an active mechanism may be playing a role in the tolerant state.

Tolerance due to signal 1 antigen presentation without signal 2 has been postulated to occur in a model in which mice exposed to Class I-like

Qa1 alloantigens in the absence of 'help' were unable to reject Qa1-bearing skin grafts (208). When help was provided at a later time, these mice were still unable to reject their grafts. This lack of response to Qa1 alloantigens was also observed *in vitro*. Similar findings in which T cells are rendered tolerant by recognition of the H-Y antigen (signal 1) in the absence of 'help' have been reported by Guerder and Matzinger (209, 210). However, direct evidence for clonal anergy has *not* been provided in these studies and active mechanisms cannot be excluded.

Active Mechanisms of Tolerance

Active mechanisms in maintaining self tolerance

Proponents of peripheral mechanisms of tolerance induction have largely favored passive (deletion/inactivation) mechanisms. There have been, however, reports in support of active (suppressive) mechanisms (211-213). An elegant experiment by McCullagh (211) examined whether the normal self-tolerant state was active or passive. Tolerance to thyroid self antigens was examined following *in utero* ablation of the the developing thyroid by exposure to ^{131}Iodine (^{131}I). Rats exposed to this agent in fetal life developed autoimmune thyroiditis when syngeneic thyroid grafts were implanted in the adult animal. The development of thyroiditis in such grafts was prevented when ^{131}I-exposed rats were parabiosed with normal syngeneic rats. If self tolerance was due solely to deletion or anergy, autoimmunity would have been observed in both animals. This experiment demonstrated that normal rats were capable of suppressing the expression of anti-thyroid reactivity, that is, the self-tolerant state was dominant over the autoimmune process.

Similarly, evidence for an active mechanism in the maintenance of self tolerance comes from experiments in which non-depleting anti-CD4 mAb, given in conjunction with mouse thyroglobulin, inhibits the development of experimental autoimmune thyroiditis (212). In this model, thyroglobulin-induced thyroiditis did not develop in anti-CD4 treated recipients and the suppression of this response could be transferred to secondary recipients; lymph node cells from antibody treated donors prevented the development of thyroiditis induced in lightly irradiated syngeneic recipients. Passive mechanisms of tolerance cannot account for these observations. Therefore, in addition to clonal deletion/anergy, active mechanisms may play a role in the maintenance of self tolerance.

Active mechanisms in allograft tolerance in adult animals

By far, most states of donor-specific tolerance to allografts induced in adult animals have implicated active regulatory mechanisms. Such active mechanisms have been postulated in tolerant states induced by CsA (214, 215), mycophenolic mofetil (formerly RS-61443) (108), or rapamycin (109), anti-donor alloantibody (140), DSBT (115, 216) or mAb directed to T cell surface molecules (141). A regulatory process has also been implicated in particular models of neonatal tolerance (103). In each of these models, the *in vitro* anti-donor reactivity of lymphoid cells from tolerant animals is similar to that of non-tolerant control animals.

In situations in which mAb are used to induce tolerance to cardiac or skin allografts, lymphoid cells from tolerant recipients were able to respond to donor alloantigens in MLC and CTL assays (123, 217, 218). Attempts to 'break' tolerance by infusion of large numbers of spleen cells or by transplanting secondary skin grafts in combination with high doses of IL-2 failed (111). These results support an active, dominant mechanism. An elegant demonstration of dominant 'infectious' tolerance was recently reported by Waldmann's group (141). Monoclonal antibody-induced tolerance to minor-mismatched skin grafts could not be broken by the infusion of normal spleen cells. Instead, co-transfer of tolerant and naive cells demonstrated that the tolerant state was conferred to the naive inoculum. Further, this inoculum conferred tolerance to a second infusion of naive spleen cells, hence the 'infectious' nature of the tolerant state. This 'infectious' tolerance was dominant, indicating that an active regulatory mechanism was involved.

An active, regulatory form of tolerance has also been implicated in tolerance to rat heart allografts, induced with CsA (219) or anti-donor antibody (220) treatment. $CD4^+$ T cells from such treated animals respond normally to donor alloantigen *in vitro* and can transfer the tolerant state to secondary irradiated rats, specifically suppressing the ability of naive cells to restore graft rejection in irradiated recipients. These findings are indicative of an active mechanism. The factors responsible for such dominant states of tolerance, however, are still speculative.

The examples cited above indicate that tolerance induced to allografts in adult animals can occur without apparent deletion or inactivation of donor-reactive T cells. Indeed, the extent to which passive or active mechanisms operate in induced allograft tolerance is controversial. These issues will be examined in this thesis by determining the nature of tolerance induction to APC-depleted islet allografts.

TOLERANCE TO APC-DEPLETED ALLOGRAFTS

The idea that 'signal 1' antigen presentation is a null event, leading to neither activation or tolerance, can be illustrated with APC-depleted allografts. Although not immunogenic, such grafts are still antigenic and are rejected when the host is immunized with donor APCs early after grafting (45, 100, 101). Such APC-depleted grafts are said to be in a '*metastable*' state relative to the host (46). However, with time after grafting, APC-depleted grafts reach a '*stable*' state in which they are no longer susceptible to rejection (46). Bowen *et al* (221) initially observed that the immunization of recipients bearing high O_2 cultured islet allografts for greater than 100 days did not lead to graft rejection. Further studies demonstrated that, with increasing time between transplantation and APC challenge, recipients of APC-depleted grafts became progressively more resistant to rejection following host immunization (116, 117). The time required for this process varies with the type of tissue transplanted (46). This resistance to rejection following host immunization with donor APCs is referred to as graft '*stabilization*' (46). The phenomenon of graft stabilization could potentially be due to: 1) a change in the antigenic composition of the graft such that it can no longer be recognized as foreign (graft adaptation) and/or 2) a change in the reactivity of the host to the graft (tolerance).

Based on the classical concept of allograft immunity, one explanation for the phenomenon of graft stabilization could be that, over time, a loss or down-regulation of MHC antigens occurs. A change in the antigenic composition of the graft was initially referred to as graft '*adaptation*' by Woodruff and Woodruff (222). They postulated adaptation as a mechanism for the long-term acceptance of thyroid allografts following transplantation to the anterior chamber of the eye and regrafting to a subcutaneous site. Alteration of graft antigens was also proposed to explain the survival and growth of tumors in allogeneic recipients following a period of organ culture (84). To determine whether adaptation could explain the resistance of APC-depleted thyroid allografts to host immunization, stable allografts were carefully removed from the host and regrafted into naive recipients, syngeneic with the original host. These thyroid grafts, following revascularization and then host immunization with donor spleen cells, were susceptible to graft destruction (116, 223). This indicated that the long-surviving graft retained recognizable antigen, although it may be argued that the surgical trauma involved in the relocation of the graft upregulated MHC antigen expression and induced graft failure.

Rather than a change occurring in the allograft itself, a second explanation for the phenomenon of graft stabilization is that there is a donor-specific change in the reactivity of the host to the graft, that is, tolerance. There is evidence that the stabilization of cultured thyroid allografts is due to donor-specific tolerance induction (116, 118). The mechanism of graft stabilization in animals bearing high O_2 cultured thyroid allografts was initially investigated by Donohoe *et al* (116). This group found that such animals accepted secondary uncultured donor-type, but not third-party, thyroid allografts. Although hyporesponsive *in vivo*, lymphoid cells from these animals had normal anti-donor cytotoxic responses *in vitro* (116). Together, these results indicated that graft stabilization was due to a state of donor-specific tolerance and that donor-reactive T cells were present in animals bearing stable thyroid allografts. Passive mechanisms of tolerance, therefore, did not appear to play a role in this model system.

Recently, La Rosa *et al* (118), utilizing animals bearing stable hyperbaric O_2 cultured thyroid allografts, demonstrated that this tolerant state was not due to the deletion of specific CTLp. CTL precursor frequencies in thyroid grafted animals were equivalent to control animals.

However, unlike the previous study by Donohoe *et al* (116), slight reductions of anti-donor CTL responses were observed both *in vivo* and *in vitro*. The addition of exogenous cytokines, in the form of Con A activated spleen cell SN, restored these responses to normal levels. This result was interpreted as the defective activation of CTLp. Though the partial block in CTL activation observed with tolerant animals could be interpreted as clonal anergy, La Rosa *et al* (118) showed that an active mechanism was involved in the maintenance of this tolerant state. They demonstrated that the tolerant state could be transferred to normal mice in parabiosis experiments. Tolerant and control animals were surgically joined so that they shared the same circulation. Each animal was grafted with cultured thyroid allografts, sharing MHC antigens with the original graft, and then the parabiosed pair was immunized with donor-type APCs. Grafts in both the tolerant and control animals were protected from rejection, indicating that the tolerant state dominated the normal immune response of the control animal. In contrast, the parabiotic union of two control animals did not protect cultured thyroid grafts from rejection following host immunization. In addition to this study, the tolerant state could block the effector function of primed donor-specific T cells *in vivo* (118). These experiments indicated that the tolerant state was dominant over the normal non-tolerant cells. Thus, the phenomenon of thyroid graft stabilization appears to be due to an active form of tolerance, however, the factors responsible for this state are not clear.

The evidence for tolerance developing in response to long-established APC-depleted islet allografts is less clear. Early reports of tolerant states were sketchy (224, 225) or tolerance was not observed at all (75, 226). The mechanism responsible for graft stabilization in this model is not clear, although an active mechanism may be involved (117).

HYPOTHESIS

A major goal of this thesis is to characterize the phenomenon of cultured islet allograft stabilization and to determine whether APC-depleted islet allografts allow the induction of donor-specific tolerance. Two issues must be resolved: 1) Is the phenomenon of graft stabilization due to the induction of donor-specific tolerance in the host? and 2) If so, is the tolerant state maintained by an active or passive mechanism?

According to the two-signal model for T cell activation, 'signal 1' antigen presentation, without provision of the second CoS signal, will not activate T cells and may be tolerogenic. Data from Jenkins and Schwartz (132, 227) indicate that such inappropriate antigen presentation can lead to T cell clonal anergy. The interpretation of this *in vitro* data, and several *in vivo* transgenic studies, might lead one to hypothesize that an APC-depleted allograft, characteristic of 'signal 1' antigen presentation, will induce T cell anergy within the host T cell repertoire. Indeed, the tolerant state developing in response to APC-depleted grafts has been cited as evidence supporting the hypothesis that antigen presentation by 'signal 1' alone leads to T cell clonal anergy (180, 227, 228). Thus, the general hypothesis tested will be: *The mechanism of tolerance induced to allogeneic tissue in an adult immune system versus in a newly developing immune system is the same.* Specifically, the hypothesis that: *'Signal 1' antigen presentation leads to clonal inactivation/anergy in vivo* will be examined. The immune response to APC-depleted allografts - a model of signal 1 antigen presentation - will be used to test these hypotheses.

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II

TOLERANCE INDUCTION TO CULTURED ISLET ALLOGRAFTS

I. Characterization of the Tolerant State[1]

The two-signal hypothesis for T cell activation implies that alloantigen alone is not the barrier to allograft acceptance (1). Rather, tissue immunogenicity is derived from metabolically active APCs resident within the graft which are capable of providing the appropriate costimulatory signals necessary for T cell induction. This model predicts that removal of donor-type APCs, or passenger leukocytes, prior to grafting will reduce or eliminate tissue immunogenicity. This theory is supported by observations that a variety of approaches designed to eliminate donor APC prior to grafting can facilitate subsequent allograft acceptance. Pretreatment with low temperature culture (2), anti-Ia (3) or dendritic cell (4) antibodies, ultraviolet irradiation (5), low pH culture (6), and perinatal islet harvesting (7) have all led to prolonged islet allograft survival with little or no host immunosuppression. This is particularly important for pancreatic islet transplantation for insulin-dependent diabetes mellitus where the risks associated with the long-term use of immunosuppressive agents may outweigh the benefits of transplantation.

Previous studies by our laboratory have shown that pretreatment of thyroid and islet tissues in 95% O_2 culture results in subsequent long-term graft survival and function in non-immunosuppressed allogeneic

[1] A version of this chapter has been accepted for publication. Coulombe MG and Gill RG. 1993. Transplantation

recipients (1, 8). In the initial post-transplant period, cultured grafts are considered to be in a metastable state relative to the recipient; the established cultured allografts are promptly rejected if the host is actively immunized with donor-type APC (9, 10). Over time, however, such recipients become progressively resistant to rejection by immunization with donor-type APC; the host develops a stable state relative to the graft (graft stabilization) (10-12).

This process of graft stabilization may be due to a change in the graft whereby the inherent vulnerability of the transplant to rejection decreases with time (graft adaptation) (13) and/or to altered immune reactivity in the host (immunological tolerance). Previous work with thyroid tissue cultured in 95% O_2 prior to allotransplantation has indicated that this 'stable' state is due to the induction of donor-specific tolerance (11, 14). However, previous studies have observed that culture of thyroids in hyperbaric oxygen results in decreased MHC expression by the graft, suggesting that tissue APC depletion may not be the sole mechanism responsible for extended allograft survival (15). Thus, questions arise as to whether survival and eventual graft stabilization of pretreated tissues in immunocompetent allogeneic hosts is due to a change in immune reactivity of the host or due to the modification of alloantigens expressed by the graft.

In this study we have extended previous results using cultured thyroid grafts by examining the phenomenon of pancreatic islet allograft stabilization. Results indicate that islet allograft stabilization is associated with donor-specific tolerance induction and not due to any apparent change in the graft itself .

MATERIALS AND METHODS

Animals. Male C57BL/6ByJ (B6, H-2^b), CBA/J (CBA, H-2^k) and BALB/cByJ (BALB/c, H-2^d) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). C.B-17 *scid/scid* (scid, H-2^d) mice were generously provided by L. Shultz and bred at the Barbara Davis Center rodent facility.

Islet preparation and transplantation. Islets were isolated from cyclophosphamide-pretreated adult mouse pancreata by collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN) digestion (16) and Ficoll purification (17). The islets were handpicked and groups of 50 islets were cultured at 37°C in an atmosphere of 95% O_2/5% CO_2 for 7 days (16). Transplant recipient BALB/c or CBA mice were rendered diabetic (blood glucose >20mM) by a single bolus iv injection of 225-275 mg/kg streptozotocin (Calbiochem, La Jolla, CA). Diabetic recipient mice were grafted with cultured islets beneath the left kidney capsule. Graft function was assessed by monitoring blood glucose values weekly with an Exactech® blood glucose meter (MediSense, Inc. Cambridge, MA). Graft rejection was defined as the first of consecutive blood glucose values above the normal range.

Assessment of graft stabilization. At various times post-transplantation, animals bearing functioning allografts were immunized with 10^5 donor-type spleen cells ip. Blood glucose was monitored three times/week following these challenges. After two weeks, normoglycemic animals were said to have stable grafts. Graft stabilization was then confirmed with a second challenge of 10^6 donor-type spleen cells.

Secondary islet grafting in long-term allograft recipients. BALB/c mice bearing functioning cultured B6 islet allografts for 90 days after transplantation received a secondary graft of 50 cultured B6 islets (as a sentinel graft for allograft immunity) under the capsule of the contralateral kidney to the primary allograft. In parallel, age-matched control BALB/c mice were also grafted with 50 cultured B6 islets. Thirty days later (day 120 after initial grafting) animals in both groups were challenged with 10^5 donor-type spleen cells. Laparotomies were performed two weeks later to examine the sentinel graft and rejected grafts were removed. Animals with intact grafts were challenged with 10^6 donor-type spleen cells and all grafts were examined after thirty days.

Assessment of graft adaptation in scid mice. Eight week old male C.B-17*scid* mice were grafted with 250 cultured B6 islets. Ninety days after grafting, animals were reconstituted with 3×10^7 BALB/c spleen cells injected ip. Thirty days after spleen cell transfer (120 days post islet grafting), recipients were challenged with 10^5 B6 spleen cells. Two weeks after challenge, grafts were examined macroscopically and histologically for evidence of rejection. Graft survival was indicated by the presence of clearly defined, well vascularized islets under the kidney capsule. Graft rejection was characterized by the lack of visible islet tissue with only residual scarring remaining at the graft site.

Assessment of tolerance by adoptive transfer. C.B-17*scid* mice were grafted with 50 cultured B6 or CBA islets as sentinel grafts for allograft immunity. One-to-two weeks after grafting, recipient mice were injected ip with 3×10^7 spleen cells from BALB/c animals bearing long-term B6 islet allografts or from age-matched control BALB/c mice. One week after spleen cell reconstitution, these animals were challenged with 10^5 B6 or CBA spleen cells ip. Grafts then were examined three weeks after challenge for macroscopic and histologic signs of rejection as described below.

Secondary thyroid grafting. Recipients of stable, primary islet grafts (as defined above) were transplanted with untreated donor-type (B6) and third-party (CBA) thyroid grafts beneath opposite poles of the right kidney capsule (contralateral to the primary islet graft). Thirty days after secondary grafting, thyroid function was determined by the ability of the thyroids to concentrate ^{125}I as described (11). Graft survival was defined by graft ^{125}I incorporation ≥5-fold the incorporation of the islet-grafted kidney, and confirmed by histologic analysis of the thyroid graft.

Histological examination of grafted tissues. Graft-bearing kidneys were removed and fixed in 10% formal saline. Paraffin sections were stained with hematoxylin-eosin and with aldehyde fuchsin (AF) to stain insulin-containing granules. Tissue sections were examined to determine the degree of tissue damage and mononuclear cell infiltration of the graft.

Statistical Analysis. The Fischer's exact test was used for comparisons between groups.

RESULTS

Variables which influence graft stabilization.

As previously reported (1, 8, 12), cultured BALB/c islet allografts functioned indefinitely in diabetic CBA recipients without a requirement for host immunosuppression (Table II-1). We then set out to determine parameters which could influence the incidence of graft stabilization, that is, the ability of the recipient to resist rejection of the established allograft after active immunization with donor-type APC. Data in Table II-1 show that the development of graft stabilization can vary with both the initial islet mass and the duration of engraftment prior to challenge. That is, increasing either the antigen dose (mass) or the time of challenge relative to grafting significantly increased the proportion of CBA animals developing a stable condition relative to the BALB/c allograft.

The proportion of animals developing graft stabilization also is influenced by the donor-recipient strain combination. Data in Figure II-1 show the time-dependence of allograft stabilization in the B6 --> BALB/c strain combination. Nearly ninety percent of recipients in this combination develop graft stabilization by 120 days post-grafting. Animals resisting rejection following a challenge with 10^5 B6 spleen cells after 120 days were given a second challenge with 10^6 B6 cells and nearly all recipients (29/30) maintained functioning grafts. Histologically, such grafts showed variable degrees of mononuclear cell accumulations around but not invading the grafted islet tissue (Figure II-2). It is notable that this mononuclear cell infiltration was challenge-dependent; cultured allografts in animals which were not challenged had essentially no visible cellular infiltrates (data not shown). Because the B6 --> BALB/c combination demonstrated a high incidence of graft stabilization, and because the BALB/c mouse was a suitable cell transfer donor for the C.B-17*scid* recipient (18, 19), this strain combination was used in the subsequent studies.

Adaptation of the islet allograft does not account for graft stabilization.

It is conceivable that the time-dependent development of graft stabilization is due to antigenic changes in the transplanted tissues, or graft adaptation, which renders the cultured allograft less vulnerable to immune destruction over time. This issue of graft adaptation was first addressed through secondary islet grafting into recipients bearing a stable primary islet graft. If resistance to rejection through host immunization was due to a change in the long-term primary graft, then a secondary cultured graft in the same animal should be vulnerable to destruction. To test this prediction, 50 cultured B6 islets were transplanted as a sentinel graft either into BALB/c mice bearing an initial functional cultured B6 islet graft 90 days after grafting, or into age-matched control BALB/c mice. Thirty days after transplanting this sentinel graft, all animals were challenged with 10^5, followed in two weeks by 10^6 B6 spleen cells. In this way, a single recipient bore both long-term (120 days) and short-term (30 days) established islet allografts at the time of challenge. Results show that all age-matched control animals rejected the sentinel cultured B6 islet graft after challenge, while the recipients with established primary B6 grafts failed to reject either the initial graft or the secondary sentinel graft (Table II-2). Histological assessment of these secondary cultured islet grafts revealed the same type of focal, non-invasive infiltrates as those found in the primary graft (Figure II-2). The acceptance of the secondary islet grafts in recipients bearing long-term primary allografts is not consistent with the notion that graft adaptation is responsible for initial graft survival and suggests that a host component is involved in the process of graft stabilization (tolerance induction).

The issue of graft adaptation was examined in a second set of experiments using C.B-17*scid* mice as allograft recipients. In order to dissociate long-term graft residence from the immune interaction with the host, cultured B6 islets were grafted into scid mice. Because scid mice are devoid of mature T or B cells, the islet graft was allowed to reside in the scid host without exposure to host lymphocytes. Ninety days after transplantation, scid mice were reconstituted with 10^8 congenic BALB/c splenocytes. Thirty days after lymphoid reconstitution (120 days post-transplantation), graft-bearing scid mice were immunized with donor-type (B6) spleen cells. Results shown in Table II-3 indicate that despite residence of the cultured graft for the same duration sufficient to generate graft stabilization (120 days), such recipients readily reject the established B6 islet allografts after challenge. We concluded from these experiments (Tables II-2 and II-3) that long-term residence of the cultured graft, *per se*, does not affect the vulnerability of the tissue to immune recognition and cannot account for graft stabilization.

Donor-specific tolerance can be transferred to scid mice.

If graft stabilization is not due to a change in the graft, then the most likely explanation for this phenomenon would be a change in the host, the generation of donor-specific tolerance induction. To test this hypothesis, we utilized the scid mouse as a transfer recipient to assess the presence of the tolerant state. For these experiments we took advantage of the finding that BALB/c lymphocytes will readily adoptively transfer immunity to Igh-congenic C.B-17*scid* mice (18, 19, Gill *et al*, unpublished results). As such, the function of lymphocytes from control or long-term grafted BALB/c mice could be examined in adoptive transfer experiments without the contribution of host-derived lymphocytes in the recipient scid mice. One to three weeks prior to adoptive transfer, scid mice were grafted with a cultured donor-type (B6) or third-party (CBA) sentinel allograft. Thirty million spleen cells from tolerant or age-matched control BALB/c mice were used to reconstitute the scid mouse and, one week following reconstitution, the animals were challenged with donor-type APC. Whereas spleen cells from control animals transferred the ability of scid mice to reject both donor and third-party cultured islet allografts, spleen cells from tolerant animals transferred the ability to reject third-party, but not donor-type islet allografts (Table II-4, Figure II-3). Thus, the tolerant state can be transferred to scid recipients in a donor-specific manner.

Partial tolerance to thyroid allografts

We next set out to determine whether the tolerance induced was tissue (islet)-specific by transplanting secondary thyroid grafts into animals tolerant of their primary islet graft. Results show that while age-matched control animals reject both donor-type (B6) and third-party (CBA) thyroid allografts, approximately 40% of the tolerant animals retained donor-type grafts and rejected third-party grafts with the remaining tolerant animals completely rejected both types of thyroid grafts. Notably, all of the tolerant animals retained their primary islet allograft whether or not the secondary thyroid graft was rejected.

DISCUSSION

The cultured islet allograft initially appears immunologically silent, inducing neither immunity nor tolerance, in the early stages after grafting. This apparent indifference of mature T cells to antigens expressed extrathymically also has been reported by Ohashi *et al* (20) in a transgenic model in which a LCMV glycoprotein antigen was expressed in pancreatic β cells. T cells in these animals were neither immune nor tolerant to the transgene product since infection with native LCMV activated host immunity leading to β cell destruction and diabetes. The ability to trigger the rejection of cultured allografts by host immunization early after grafting (9, 12, 21) indicates that, although the cultured allograft does not trigger a rejection response, it does express recognizable alloantigens which can serve as a target for immune destruction.

With time, however, immunization with donor-type APC no longer results in graft rejection. This phenomenon of graft stabilization in long-term cultured thyroid or islet allograft recipients has been previously reported (10-12, 22). However, the proportion of animals developing this stable condition in these studies varied. We have characterized graft stabilization in a murine islet allograft system and have found that the tissue mass transplanted, the time between transplantation and challenge, and the donor-recipient combination all influence the incidence of graft stabilization.

A key issue was to determine whether the generation of graft stabilization was due to a progressive change in the antigen expression by the graft (graft adaptation) or due to a change in the immune reactivity of the host (tolerance induction). The former possibility is especially important to consider since some forms of tissue culture may facilitate graft acceptance through the modulation of graft antigens (15, 23). However, grafting experiments in scid mice show that the long-term residence of the graft, independent of exposure to host lymphocytes, is not sufficient to decrease the vulnerability of the cultured graft to immune recognition. Also, animals which develop into the stable state resist rejecting secondary short-term islet grafts. Taken together, these experiments indicate that adaptation of the graft to the host (for example, through modulation of graft antigens) cannot account for graft stabilization. An alternate explanation is that graft stabilization is due to a change in the host, the induction of tolerance, a conclusion which is supported by experiments that show that donor-specific tolerance to cultured islet allografts can be transferred to scid recipient mice. As such, these results are consistent with previous studies in which graft stabilization developing in animals receiving cultured thyroid allografts was shown to be associated with donor-specific tolerance (11) and later shown to involve a dominant, regulatory form of tolerance (14). The use of the scid mouse as an adoptive transfer recipient should allow us to further dissect the nature of tolerance which develops in this system.

The generation of donor-specific tolerance in response to cultured islet allografts implies that some form of recognition of donor antigens was required by the recipient for the induction of tolerance. Ultrastructural examination of thyroids and islets cultured in 95% O_2 revealed that donor class II^+ APC and vascular endothelium degenerate in this form of treatment, indicating that these grafts are comprised almost entirely of MHC class I^+, class II^- tissue parenchymal cells (24). Due to a lack of APCs in the cultured grafts, the most likely modes of host interaction with donor antigens would be either through direct recognition of parenchymal cells of the graft and/or through indirect presentation of processed donor antigens by host-derived APCs. In either case, these results imply that the cultured graft is not immunologically ignored, but rather that some form of donor antigen recognition generates a response which leads to allograft tolerance rather than immunity.

One hypothesis is that an interaction with islet parenchymal cells, which are devoid of costimulatory activity, delivers a tolerogenic signal to alloreactive T lymphocytes rendering these cells refractory to immunogeneic stimuli (25, 26). This concept is not supported by further studies which show that animals which develop tolerance to cultured islet allografts retain normal anti-donor reactivity, a finding which is more consistent with the view that tolerant animals develop an active, regulatory form of tolerance *in vivo* (27). However, there remains the possibility that a tissue (islet)-specific form of tolerance develops in these animals; a notion that deserves attention since some recipients tolerant to islet allografts can still reject secondary donor-type thyroid grafts. However, other studies show that tolerant animals retain the ability to react to donor islet tissue both *in vitro* and *in vivo* (27). We would propose that the ability of some tolerant animals to reject secondary thyroid grafts involves a response to class II MHC expression on the thyroid graft (23), an important alloantigen to which the recipient would not be tolerant given that the cultured islet tissue does not express class II MHC (24). Thus tolerance may not be tissue-specific as reflected by islet-specific peptide antigens, but rather specific for allogeneic class I and not class II MHC.

Finally, it is noteworthy that tolerant animals are not completely unresponsive *in vivo*; upon challenge with donor-type APC there is a modest, non-destructive mononuclear cell accumulation around the graft. It will interesting to examine the function of cells derived from these non-destructive lesions for evidence of potential altered donor reactivity.

TABLE II-1. Islet allograft stabilization is influenced by both initial islet mass and the time between grafting and donor-type APC challenge.

Group Number	Number of Islets Grafted	Time of APC Challenge(days)	Graft Survival Total Challenged
I	350	120	5 / 20 (25%)
II	350	200	7 / 10 (70%)
III	450	120	8 / 10 (80%)

CBA mice were grafted with varying numbers of cultured BALB/c islets and were challenged with 10^5 donor spleen cells (as a source of APCs) at the times indicated. Normoglycemia following challenge (resistance to rejection) was indicative of graft stabilization.

I vs II: $p = 0.024$, II vs III: $p = 0.500$, I vs III: $p = 0.006$.

TABLE II-2. Secondary donor-type islet grafts are protected in recipients bearing long-term cultured allografts.

Group[a] Number	Functional Islet Graft	Sentinel Graft	APC Challenge	Sentinel Graft Survival
I	+	+	+	19 / 19[b]
II	--	+	+	0 / 11

[a] Ninety days after initial grafting with cultured B6 islets, BALB/c recipients were grafted with a secondary B6 cultured islet graft under the kidney capsule contralateral to the primary established graft (group I). Age-matched control animals (group II) were grafted with B6 islets at the same time. Thirty days after this secondary grafting, all animals were challenged with 10^5 B6 spleen cells as a source of donor-type APCs. Normoglycemia was indicative of initial allograft function. All grafts were examined macroscopically and histologically to determine graft survival.

[b] I vs. II: $p < .0001$

TABLE II-3. Long-term residence of a cultured islet allograft is not necessary or sufficient for graft stabilization.

Group[a] Number	**BALB/c splenocyte Reconstitution**	**APC Challenge**	**Graft Survival**
I	+	--	3 / 3[b]
II	+	+	0 / 5

[a] C.B-17*scid* mice were grafted with 250 cultured B6 islets on day 0. Ninety days after transplantation, these mice were reconstituted with 3 x 10^7 normal BALB/c spleen cells. Thirty days after spleen cell reconstitution (120 days post-transplantation) recipients were immunized with 10^5 followed in two weeks with by 10^6 B6 spleen cells (as a source of donor-type APC). Graft survival was assessed by macroscopic and histologic inspection of the grafted tissues.

[b] Group I vs II: $p = 0.018$

TABLE II-4. Sentinel islet allograft survival in scid mice reconstituted with spleen cells from tolerant or age-matched control BALB/c mice.

Spleen cell Reconstitution	APC Challenge	Sentinel Graft Survival	
		Donor (B6)	Third-Party (CBA)
None	+	4 / 4	3 / 3
Tolerant	+	16 / 18 [a,b]	1 / 7 [b]
Control	+	2 / 14 [a,c]	0 / 6 [c]
Control	--	2 / 2	ND

C.B-17*scid* mice were grafted with 50 cultured donor (B6) or third-party (CBA) islets one week prior to reconstitution with 3×10^7 spleen cells from tolerant or control mice. One week following reconstitution, the animals were challenged with 10^5 donor-type (B6 or CBA) spleen cells. Three weeks following this challenge, the grafts were examined histologically.

[a] $p = 3.01 \text{ X } 10^{-5}$

[b] $p = 7.49 \text{ X } 10^{-5}$

[c] $p = 0.479$

TABLE II-5. Survival of thyroid allografts in recipients of long-term cultured islet allografts.

Recipient	Cultured Islet Allograft Survival	Thyroid Graft Survival Donor (B6)	 Third-Party (CBA)
Tolerant	25 / 25 [a]	11 / 25 [a,b,c]	0 / 25 [b]
Age-matched Control	-----	0 / 21[c]	0 / 21

BALB/c recipients of stable islet allografts or age-matched control animals received thyroid lobes from donor (B6) and third-party (CBA) animals beneath opposite poles of the right kidney. Islet survival, assessed by normoglycemia, and thyroid function (^{125}I uptake $\geq$ 5 times background) were confirmed histologically.

[a] $p = 1.4 \times 10^{-6}$

[b] $p = 3.2 \times 10^{-4}$

[c] $p = 8.0 \times 10^{-4}$

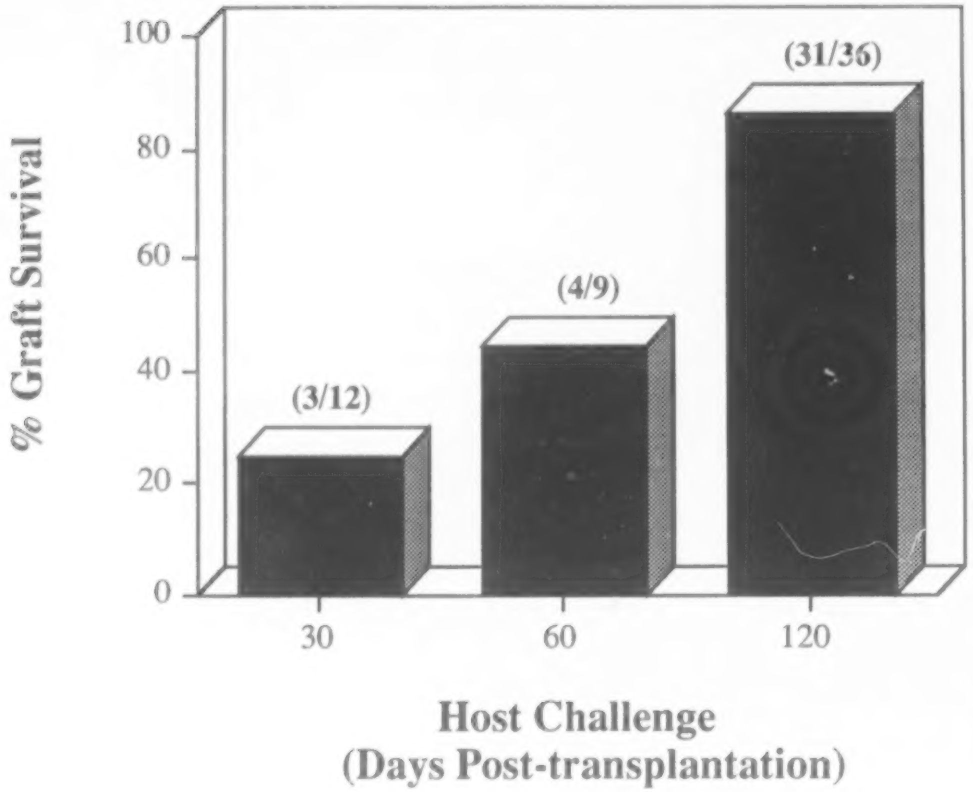

FIGURE II-1. ***Graft stabilization is a time dependent process.*** BALB/c mice were grafted with 400 cultured B6 islets on day 0 and then immunized with 10^5 B6 spleen cells at the times indicated. Graft survival was assessed by both function (normoglycemia) and histological examination.

30d vs 60d: $p = 0.319$
60d vs 120d: $p = 0.016$
30d vs 120d: $p = 0.0002$

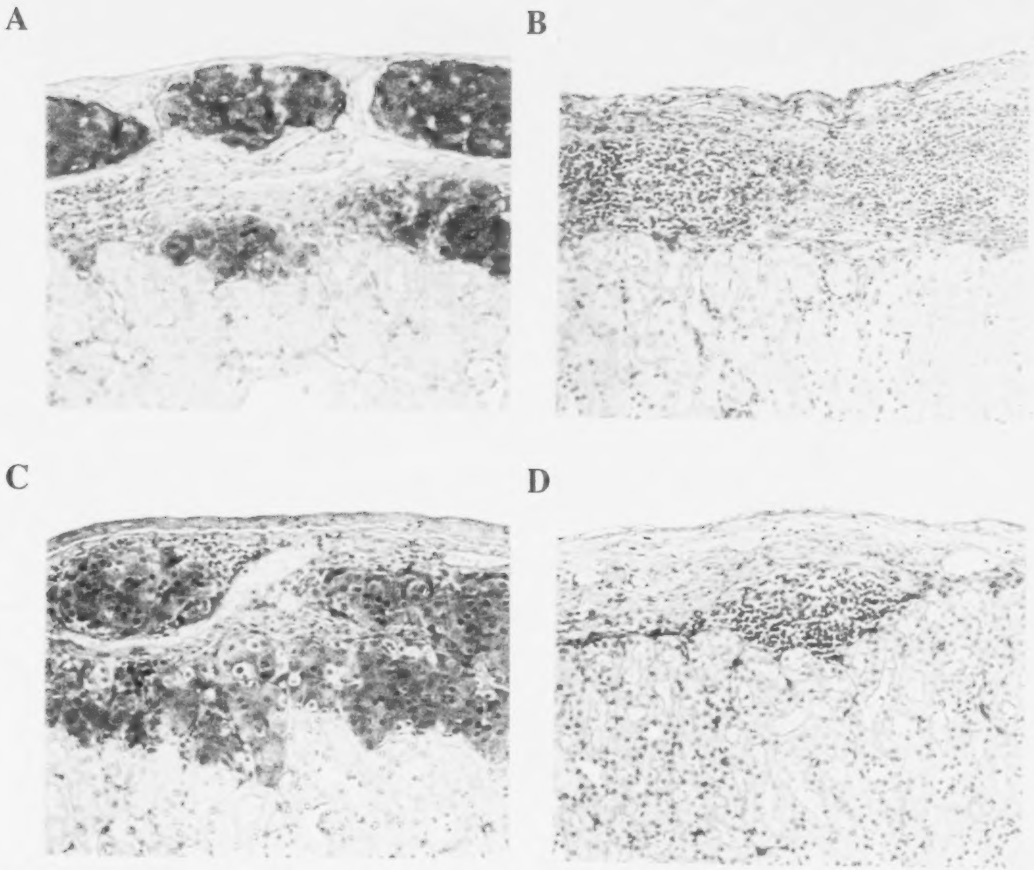

FIGURE II-2. *Graft stabilization results in the survival of primary and secondary cultured B6 allografts in BALB/c recipients.* **A**: Primary functional cultured B6 islet allograft from a BALB/c recipient challenged 120 days post-grafting with 10^5, followed in two weeks by 10^6, B6 spleen cells. AF staining shows well-granulated islet tissue and surrounding focal mononuclear cell accumulation. **B**: B6 islet allograft from a BALB/c recipient challenged 30 days post-grafting with 10^5 spleen cells. Note complete graft destruction with residual mononuclear cell infiltration. Sentinel cultured B6 islet allograft in BALB/c mouse bearing a primary islet graft for 90 days (**C**) and in a naive age-matched control BALB/c recipient (**D**). Both animals were challenged with B6 APC 30 days after graft placement. AF-staining shows well-granulated islet tissue despite visible focal mononuclear cell accumulation in the recipient bearing a primary graft while the naive recipient acutely rejected a similar graft after challenge (original magnification x100).

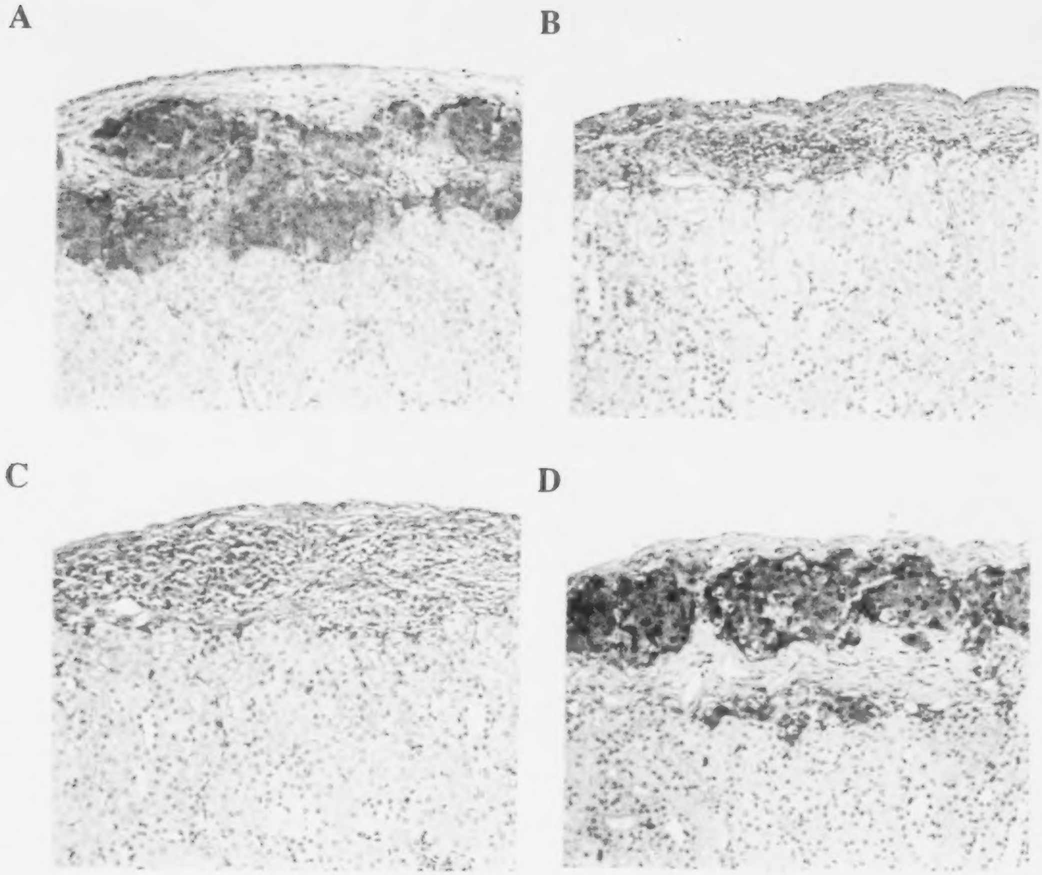

FIGURE II-3. *Donor-specific tolerance can be transferred to scid mice.* **A**: B6 sentinel islet allograft in scid mouse following reconstitution with $30x10^6$ spleen cells from tolerant animals and challenge with 10^5 B6 spleen cells. AF staining demonstrates intact islet tissue with insulin granules. **B**: B6 sentinel islet allograft in scid recipient reconstituted with control BALB/c spleen cells and challenged with B6 spleen cells. AF staining shows complete destruction of islet tissue and residual mononuclear cell infiltration. **C**: Complete destruction of a third-party CBA sentinel islet allograft in a scid recipient reconstituted with tolerant spleen cells and challenged with 10^5 CBA spleen cells. Similarly, CBA sentinel grafts were destroyed in scid mice reconstituted with control BALB/c spleen cells and challenged. **D**: A sentinel CBA islet allograft remains intact and well-granulated in an unreconstituted scid recipient. (original magnification x100)

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III

TOLERANCE INDUCTION TO CULTURED ISLET ALLOGRAFTS

II. Status of Anti-Donor Reactivity in Tolerant Animals[1]

It is well established that T cell activation requires two signals: 1) occupancy of the T cell receptor, and 2) an appropriate costimulatory signal (1). One proposition of this model was that antigen (signal 1) alone, devoid of costimulator activity, was not merely a null event but rather delivered a negative signal to the responsive T cell rendering the cell refractory to an immunogenic stimulus. This form of T cell inactivation - or anergy - was demonstrated through experiments where the delivery of signal 1 alone could induce anergy in certain T cell clones (2). Anergy induced by signal 1 alone has also been implicated in several transgenic mouse models in which foreign MHC antigen is expressed on nonlymphoid peripheral tissues via tissue-specific promoters (3, 4). In these transgenic studies, the transgene is expressed during the ontogeny of the immune system. Our own interest is whether a similar form of tolerance occurs in the adult animal exposed to allografts which are devoid of costimulator activity.

The ability to reduce tissue immunogenicity with pretransplant regimens thought to selectively eliminate or inactivate APCs from the graft (1) offers a unique way of exposing immunocompetent adult animals to transplantation antigens. Allogeneic tissue, normally rejected within a few weeks, can survive indefinitely in nonimmunosuppressed recipients if if the tissue is cultured in 95% O_2 (5%CO_2) at 37°C for one week prior to transplantation (5). Such cultured grafts do not elicit a rejection response but do express recognizable alloantigens in that immunization with donor-type APC triggers acute rejection of the established graft (6-8). Over time, however, these grafts become resistant to rejection by immunization with donor APC and reach a stable state relative to the host (graft stabilization) (7-9). In the previous study, we have characterized islet allograft stabilization by showing that this state is associated with donor-specific tolerance induction *in vivo* (8). This model allows us to study peripheral mechanisms of tolerance to extrathymically expressed alloantigens and, since cultured allografts supply signal 1 only, we are able to determine whether such antigen presentation results in the deletion/anergy of donor-reactive T cells. Specifically, we test the hypothesis that tolerance developing to cultured allografts in immunocompetent adult animals is equated with the elimination / inactivation of donor-reactive T cells by examining anti-donor reactivity both *in vitro* and *in vivo*.

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[1] A version of this chapter has been accepted for publication. Coulombe M and Gill RG. Transplantation. 1993

MATERIALS AND METHODS

Animals. Six to 8 week old male C57BL/6ByJ (B6, H-2^b), CBA/J (CBA, H-2^k) and BALB/cByJ (BALB/c, H-2^d) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). C.B-17 *scid/scid* (scid, H-2^d) mice were generously provided by L. Shultz and bred at the Barbara Davis Center.

Cell lines. P815, a DBA/2 (H-2^d) mastocytoma, and EL-4, a C57BL/6N (H-2^b) lymphoma, were maintained by serial passage in Dulbecco's modified Eagle's minimal essential medium (Gibco) supplemented with

10% heat-inactivated fetal bovine serum. WEHI-164, a TNF-sensitive cell line (10) and IL-3-dependent FDC-P1 cells (11) were maintained as above. FDC-P1 cells were supplemented with IL-3 containing WEHI-3 SN.

Islet preparation and transplantation. Islets were isolated from cyclophosphamide-pretreated donor B6 adult mouse pancreata by collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN) digestion (12) and Ficoll purification(13). The islets were handpicked and cultured at 37°C in an atmosphere of 95% O_2 for 7 days. BALB/c mice, rendered diabetic (blood glucose >20mM) by the iv injection of 225 mg/kg streptozotocin (Calbiochem, La Jolla, CA), were used as allograft recipients. Diabetic recipients were grafted on day 0 with 400 cultured islets beneath the kidney capsule. Graft function was assessed by monitoring blood glucose values weekly with an Exactech® blood glucose meter (MediSense, Inc. Cambridge, MA). At 120 days post-transplantation, animals bearing functioning allografts were immunized with 10^5 donor spleen cells ip. Blood glucose was monitored 2-3x per week following this challenge. After two weeks, normoglycemic animals were said to have stable grafts. Graft stabilization was confirmed by the ability of these animals to resist graft rejection following a second challenge of 10^6 donor-type spleen cells.

Mixed leukocyte cultures (MLC). The mixed leukocyte reaction was established by mixing BALB/c lymph node (LN) or spleen cell responders ($2x10^5$) with 2000R-treated B6 splenic stimulator cells ($3x10^5$) in a total of 0.2 mL cultures in 96-well flat-bottom plates (Linbro). Cells were cultured in Eagle's Minimal Essential Medium (Gibco) supplemented with

10% fetal calf serum, 10^{-5}M 2-mercaptoethanol, and antibiotics and incubated at 37° C in 10%CO_2 in air. Proliferative responses were determined by pulsing cultures with 1.25 µCi [^{3}H]-thymidine for 6h on days 3, 4 and 5 of primary culture. For CTL assays and for expansion of primary activated alloreactive T cells, primary MLC were established in 24-well plates (Falcon) with 2x10^6 LN or spleen cells as responders and 3x10^6 2000R-treated splenic stimulator cells.

Limiting dilution analysis. Cytotoxic T cell precursor (CTLp) frequencies were determined as follows: Limiting numbers of LN or spleen cells (32 replicates / group) from tolerant or age-matched control animals were cultured in 96 well plates (Linbro) with 0.5 x 10^6 irradiated B6 spleen cells and Con A supernatant (SN). On days 4 and 6, medium was removed from each well and fresh Con A SN was added. On day 7, 100 µl of cells from each well were assayed for cytotoxic activity in the ^{51}Cr release assay described below. Cultures with greater than 3SD of lytic activity over spontaneous release were scored positive. CTLp frequency was determined using the Poisson distribution.

^{51}Cr-release assay. Varying numbers of effector T cells, harvested on day 5 of MLC, were incubated with 10^4 ^{51}Cr-labelled tumor target cells for 4h at 37°C in 10% CO_2. Supernatants were harvested and ^{51}Cr release was detected on a gamma counter. Cytotoxic activity is expressed as $\log_{10}$ cytotoxic units (CU) per culture as previously described (14).

Triggering of cytokine release from activated T cells. Activated T cells were prepared by four day MLC followed by a three day expansion (seven days total culture) in IL-2-containing media as previously described

(15). Such expanded T cells display undetectable cytokine production unless triggered with their specific antigen. Fixed numbers of responding T cells were triggered to produce cytokines by varying numbers of UV-irradiated target cells as described (15). In some experiments, UV-irradiated islet cells, obtained by the trypsin digestion of isolated B6 islets (16), were used as target cells. Cultures were incubated for 6h in 10% CO_2 after which supernatants were removed for lymphokine assays.

Cytokine Assays

(a) T Cell growth factor (TCGF/IL2) assay. Test SN was measured for TCGF activity by the capacity to maintain proliferation of Con A blasts. Con A blasts were added to varying dilutions of the test SN and incubated for 18h at 37°C. The cultures were then pulsed with 1.25 μCi $[^3H]$-thymidine, incubated for 5h and measured for $[^3H]$-incorporation. This assay does not distinguish IL-2 from IL-4 (unpublished observations).

(b) IL-3 Assay. Test SN was measured for IL-3 activity by the ability to maintain proliferation of FDC-P1 cells, an IL-3-dependent cell line. These cells were added to dilutions of supernatant and incubated for 48h at 37°C. Proliferation was assayed by an MTT (3-[4,5-Dimethlythiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) colorimetic method (17). Briefly, 50 μg of MTT was added/well. After 2h at 37°C, HCl-isopropanol was added and formazan crystals were dissolved by rapid mixing. Optical density was measured on a Dynatech MR 600 Microplate reader.

(c) Gamma-Interferon (IFN-γ) assay. IFN-γ in culture SN was detected with a solid phase enzyme immunoassay (Genzyme mouse interferon-γ ELISA, Genzyme Corporation, Boston, MA). Quantities of IFN-γ in test SN were estimated from a standard curve.

(d) TNF assay. Test SN was assessed for TNF activity by the ability to kill a TNF-sensitive tumor cell line (WEHI-164). Cells were incubated in dilutions of test SN for 48h after which cell viability was assessed by the MTT colorimetric method described above.

Adoptive transfer of primed T cells *in vivo*. T cells from tolerant and control mice, activated in MLC (T'), were clonally expanded in media containing IL-2 as SN from Con A-activated spleen cells as described (15). On day 7 after primary MLC, 10^6 T' cells were embedded in a recipient-derived blood clot and implanted adjacent to an established sentinel donor-type B6 or syngeneic BALB/c islet graft residing in a normal BALB/c for 1-3 weeks. Grafts were examined two weeks after T' implantation.

Histological Examination. Islet-grafted kidneys were fixed in formalin for 24-48h and then transferred to 70% ethanol. Paraffin sections were stained with hematoxylin-eosin or aldehyde fuchsin (AF) to detect insulin-containing granules.

Statistical Analysis. The Fischer's Exact test was used for analyses of *in vivo* studies.

RESULTS

In vitro anti-donor reactivity of tolerant animals

Data from the preceeding report indicated that BALB/c mice bearing long-term (>120 days) established cultured B6 islet allografts develop a state of donor-specific tolerance (8). Tolerance was determined by: 1) Resisting triggering of graft rejection after challenges of 10^5, followed in two weeks with 10^6, donor-type (B6) spleen cells, and 2) Maintenance of a secondary sentinel B6 graft under the opposite kidney to the primary established islet graft (8). Our goal here was to determine whether there was any detectable alteration in the anti-donor response in these animals as assessed by a variety of standard immunological assays. For each type of assay, spleen and LN cells were obtained from BALB/c mice tolerant to cultured B6 islet allografts or from age-matched control BALB/c mice as responders.

We first determined whether there was any detectable impairment of the proliferative response to donor-type B6 stimulator cells. Spleen and LN cells from each animal and the appropriate control were stimulated in MLC with γ-irradiated B6 spleen cells. Proliferative responses of tolerant and control cultures were assessed on days 3, 4 and 5. Data in Figure III-1 show a typical experiment from a large number of assays. Peak responses did not differ between tolerant and control cultures although spleen cell cultures from tolerant animals often appeared somewhat primed relative to control cultures in that the response peaked on day 4 rather than day 5.

We next set out to determine whether donor-specific MHC class I-reactive T cells from tolerant animals demonstrated any impaired reactivity relative to control animals. This is an important issue since the cultured islet tissue to which the recipient was tolerized expresses only detectable class I and not class II MHC antigens (18). Further, other studies have shown that the class I-reactive $CD8^+$ T cell is the essential cell involved in the rejection of cultured islet allografts (19). For these reasons we determined whether donor-reactive T cells from tolerant animals could respond to donor-type class I^+/class II^- target cells (EL-4 tumor cells). We first determined whether tolerant animals could generate cytotoxic activity against the donor antigens. Data in Figure III-2 show that there was no difference in the ability of cells from tolerant and control animals to lyse donor MHC class I-bearing EL-4 tumor targets *in vitro*. A more subtle assessment of CTL activity was performed by determining the CTL precursor frequency of donor-reactive T cells from tolerant animals. Data in Table III-1 show that there was no difference in CTLp between tolerant and control animals for either LN or spleen responders.

Another important feature of the potentially graft-destructive T cell is the ability to produce cytokines. This is illustrated by our previous study showing that islet graft rejection by $CD8^+$ T cells is a cytokine-dependent process (20). It is possible that the bulk proliferative and cytotoxic responses are intact in tolerant animals but that there is impaired cytokine production by the class I-reactive T cell. Data in Figure III-3 show representative results from several experiments indicating that LN cells from tolerant animals can release IL-2, IL-3, and TNF in response to EL-4 tumor cells in quantities comparable to cells from age-matched control animals. It is notable that very little IL-2 is produced by the class I reactive T cells in either tolerant or control BALB/c mice, consistent with our previous unpublished results. There were no differences between spleen or LN cell populations in any of these assays (data not shown). Other experiments (Figure III-4) show that tolerant and control animals produce comparable amounts of IFN-γ, a key cytokine implicated in islet allograft rejection (20).

Primed, donor-reactive T cells from tolerant mice respond to tissue-specific (islet) antigens in vitro and in vivo.

Results described above indicated that tolerant animals responded as well as control animals to donor-type MHC class I-bearing target cells as assessed by proliferative, cytotoxic, and cytokine reactivity. However, it is certainly possible that a tissue (islet)-specific subset of the total donor-reactive repertoire, such as T cells specific for islet-derived peptides, was eliminated or inactivated in tolerant mice. Such cells could be important for the rejection response *in vivo* and yet not be reflected in bulk assays *in vitro*. The possible role for tissue-specific tolerance was tested in two types of experiments. In the first experiment, we tested whether *in vitro* activated LN cells derived from tolerant animals were able to respond to islet cells as targets *in vitro*. Data in Figure III-5 show the ability of primed, donor-reactive T cells to release IL-3A in response to dissociated B6 islet cells as antigen. This approach was taken because our IL-3 assay is very sensitive to low levels of antigen, an assay suitable to measure cytokine production in response to islet cells targets which express quite low levels of class I MHC (21). Although these results suggest that islet-reactive T cells are not eliminated in tolerant animals, it can be argued that the affinity or effector function (such as the ability to release other cytokines) of the islet-reactive T cell pool is altered in tolerant mice. Because *in vitro* conditions are often less stringent than what may occur *in vivo*, we then asked whether tolerant cells generated in response to donor-type spleen cells and clonally expanded *in vitro* were capable of destroying a B6 islet graft *in vivo*. Activated donor-reactive T cells from tolerant or age-matched control animals were placed adjacent to sentinel cultured B6 islet grafts established in normal BALB/c recipients. The results show that, in all cases, activated T cells from both control and tolerant animals efficiently destroyed the sentinel B6 islet graft over the two week observation period (Table III-2, Figure III-6). This graft destruction was not due to non-specific inflammatory damage induced by grafting the primed T cells in that syngeneic BALB/c grafts were unaffected by the transferred T cells.

DISCUSSION

T cell tolerance can be induced by two fundamentally distinct processes: 1) passive mechanisms, whereby the antigen-responsive T cell is eliminated or inactivated, and 2) active mechanisms, whereby the function of the antigen-responsive T cell is regulated by other components of the immune system. Both of these processes presumably are the result of some form of antigen-specific encounter resulting in the tolerant state. Clonal deletion of self-reactive T cells in the thymus has been a well-documented phenomenon (22, 23). Nondeletional mechanisms involving the inactivation of potentially reactive T cells may also exist for the induction of tolerance to extrathymic antigens (3, 4). However, McCullagh recently has provided evidence that self-tolerance may also involve the active regulation of potential anti-self reactivity (24). As such, the relative contribution of passive and active mechanisms in these models of self-tolerance during the ontogeny of the immune system remains a matter of some controversy. The role of such mechanisms for the induction of allograft tolerance in the adult animal is even less clear. In this study we set out to examine whether tolerance induction to cultured (APC-depleted) pancreatic islet allografts was associated with passive or active regulatory mechanisms.

In the preceeding paper, we demonstrated that BALB/c recipients bearing long-term established cultured B6 islet allografts developed a state of donor-specific tolerance (8). An intriguing hypothesis derived from this result is that the APC-depleted graft, devoid of costimulator activity, delivers a negative signal to the graft-reactive T cell (1,2). This proposition would predict that donor-reactive T cells which encounter alloantigens on non-stimulatory cells, such as islet parenchymal cells, would be rendered refractory to subsequent immunogenic stimuli. However, the current results do not support such a model in that both tolerant and control animals respond equally well to donor APCs in a variety of immunologic assays. A possible explanation for the disparity between *in vivo* and *in vitro* reactivity to donor antigen is that T cells reactive to spleen peptides in the context of H-2^b MHC antigen provide the bulk of the *in vitro* response and may mask a deleted or inactivated subpopulation of T cells specific for islet peptides in association with H-2^b. T cell reactivity to tissue-specific peptides presented in the context of MHC antigen has been reported (25). An alternative possibility is that T cells with high affinity for alloantigen may have been deleted or inactivated leaving lower affinity cells which can function *in vitro* when provided with 'help' but are incapable of responding *in vivo* (26). We can exclude both of these possibilities since: 1) *in vitro* activated T cells are capable of secreting lymphokines (IL-3) in response to islet cell targets and 2) *in vitro* activated T cells are capable of destroying donor-type islet allografts *in vivo*. Further evidence against tolerance developing to a tissue-specific peptide comes from previous results (8, 27) which demonstrate that a significant proportion of animals tolerant to a cultured islet allograft can accept donor-strain thyroids while rejecting third-party strain thyroid allografts .

These results indicate that mature, immunocompetent animals can develop a non-deletional form of tolerance to extrathymic alloantigens. Several nondeletional mechanisms for tolerance developing to antigens expressed only in the periphery have been proposed including 1) clonal inactivation (anergy), 2) down-regulation of the T cell receptor and CD8 (28), and 3) the absence of appropriate T cell 'help' (26). Further, several groups have used the rat insulin promoter to direct the expression of foreign Class I (29,30) or Class II (31-34) MHC antigens to the pancreatic β cell in order to examine extrathymic tolerance. In some of these models, tolerance to the transgene expressed on β cells was demonstrated both *in vivo* and *in vitro* (30,32). It is important to note that low-level expression of the transgene in the thymus may contribute to tolerance induction in some transgenic models (35,36). In other transgenic models of extrathymic tolerance, including models in which allo-MHC antigens are expressed in β cells (33,34), in hepatocytes via the metallothionein promoter (37) and in exocrine pancreas via the elastase promoter (38), animals demonstrated functional tolerance *in vivo* despite reactivity to the transgene product *in vitro*. This disparity between *in vitro* and *in vivo* phenomena have also been reported in some models of adult (9, 39, 40) or neonatally induced (41) transplantation tolerance.

The nature of tolerance to extrathymic antigens in transgenic mice is generally attributed to the elimination or functional impairment of reactive T cells. As such, these models suggest a passive form of tolerance without invoking a requirement for regulation of the response imposed by extrinsic immune components. However, our own results suggest that adult animals develop tolerance to cultured islet allografts *in vivo* despite bearing donor-reactive T cells which are quantitatively and qualitatively indistinguishable from control cells in their ability to respond to donor antigens by the criteria examined. As such, these results are not consistent with a passive form of tolerance but rather are more consistent with the hypothesis that an active, regulatory form of tolerance develops in these animals. Such a hypothesis is consistent with the other results whereby a dominant regulatory form of tolerance develops in recipients of cultured thyroid allografts (42) or in animals rendered tolerant to skin allografts by monoclonal antibody therapy (43).

In conclusion, our interpretation of these results is that tolerance induction to cultured islet allografts does not develop from a direct encounter of responsive cells with grafted cells leading to the subsequent paralysis of donor-reactive T cells. If this interpretation is true, then we must consider alternate forms of donor antigen recognition which may lead to tolerance induction. Since direct recognition of alloantigens on grafted cells does not appear to explain the generation of tolerance, then we would postulate that the indirect presentation of graft antigens by host APC may be involved in the generation of regulatory T cells which may interfere with the generation of the graft-specific T cell response *in vivo*. Such a model would predict that a $CD4^+$ regulatory cell would be involved in the maintenance of induced allograft tolerance, a finding consistent with the observations of Hall *et al* (44, 45).

TABLE III-1. CTLp frequencies of lymph node and spleen cell populations from tolerant and age-matched control animals.

Exp. No.	**Tolerant LN**	**Control LN**
1	1 / 3026	1 / 2325
2	1 / 2195	1 / 2115
3	1 / 1599, 1 / 2240	1 / 2656
Exp. No.	**Tolerant Spleen**	**Control Spleen**
4	1 / 3379	1 / 4800
5	1 / 7383	1 / 5641
6	1 / 1044	1 / 1933

Limiting numbers of lymphoid cells were cultured with irradiated B6 spleen cells and Con A SN. Cytotoxic activity from each well was measured in a ^{51}Cr release assay and precursor frequencies were determined using the Poisson distribution.

TABLE III-2. Survival of established donor (B6) or syngeneic (BALB/c) sentinel islet grafts following transfer of *in vitro* activated (BALB/c anti-B6) T cells derived from tolerant or control BALB/c mice.

In vitro activated BALB/c anti-B6 T cells	**Sentinel Graft Survival** **Donor**	 **Syngeneic**
Control	0 / 18	4 / 4
Tolerant	0 / 9	4 / 4

10^6 activated T cells were placed adjacent to an established cultured B6 or BALB/c sentinel graft. Grafts were examined histologically two weeks later.

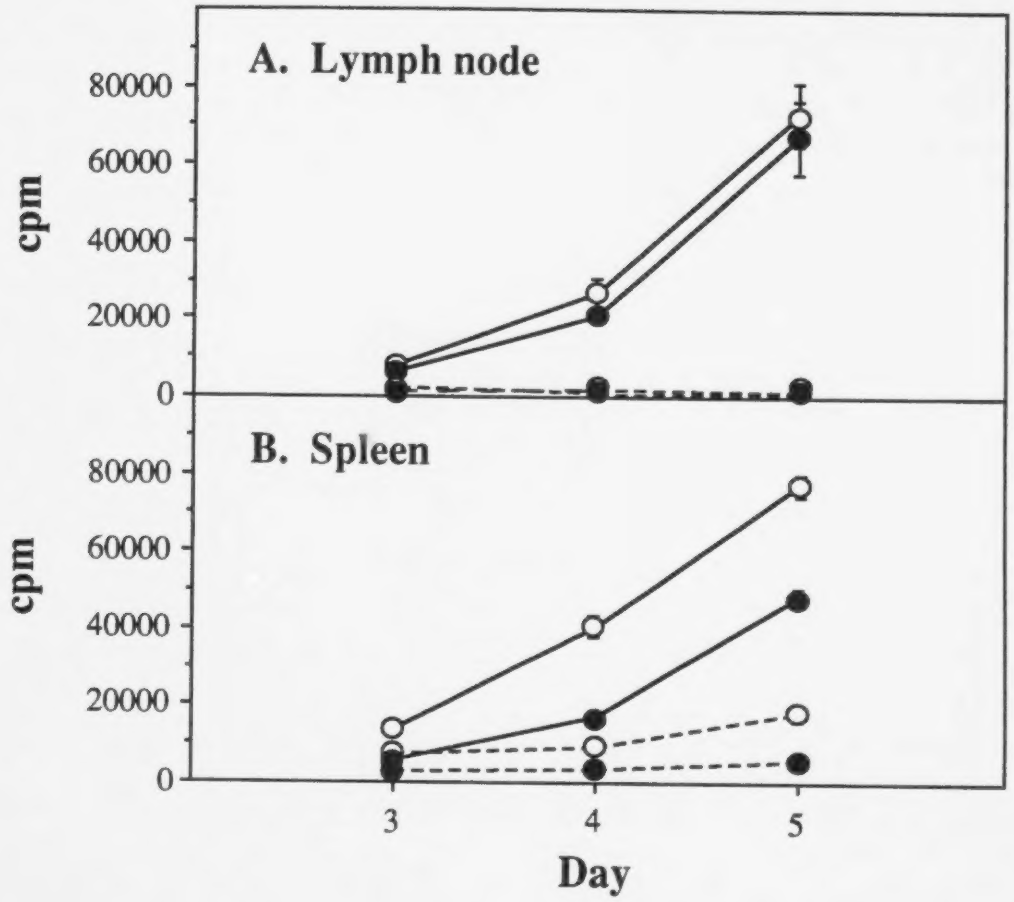

FIGURE III-1. Lymphoid cell populations from tolerant animals proliferate in response to donor (B6) antigen. Lymph node (**A**) or spleen cell (**B**) populations ($2x10^5$ / well) from tolerant (○) or age-matched control (●) BALB/c mice were incubated with (—) or without (– -) $3x10^5$ irradiated B6 spleen cells. On days 3, 4 and 5, cultures were pulsed with [^{3}H]-thymidine and harvested 6h later.

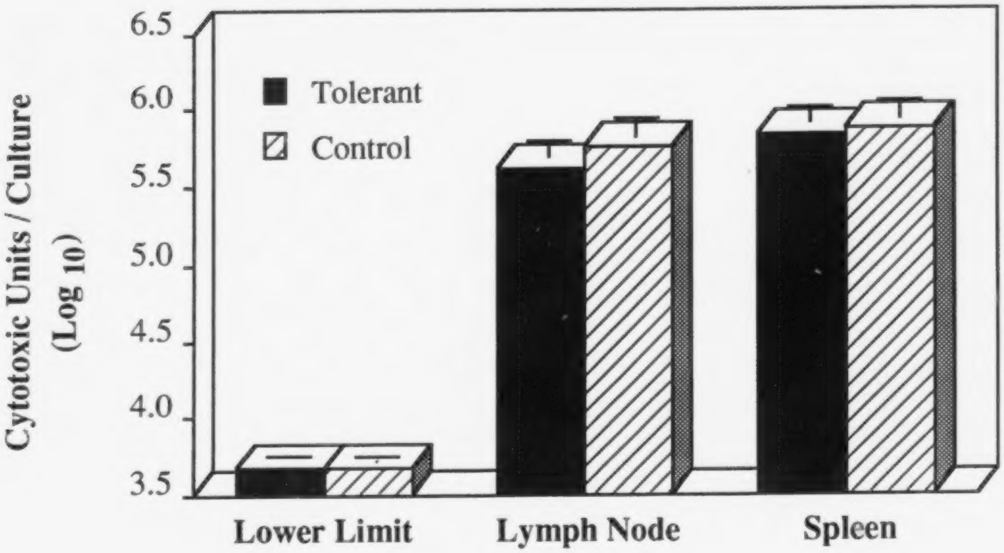

FIGURE III-2. ***Anti-donor cytotoxic responses of LN cells derived from tolerant or age-matched control animals.*** LN cells activated with B6 stimulator cells were harvested on day 5 and assessed for cytotoxicity against donor Class I antigen bearing targets (EL-4, H-2^b) in a 4h ^{51}Cr release assay. Reactivity above background levels was not observed when syngeneic (P815, H-2^d) targets were used.

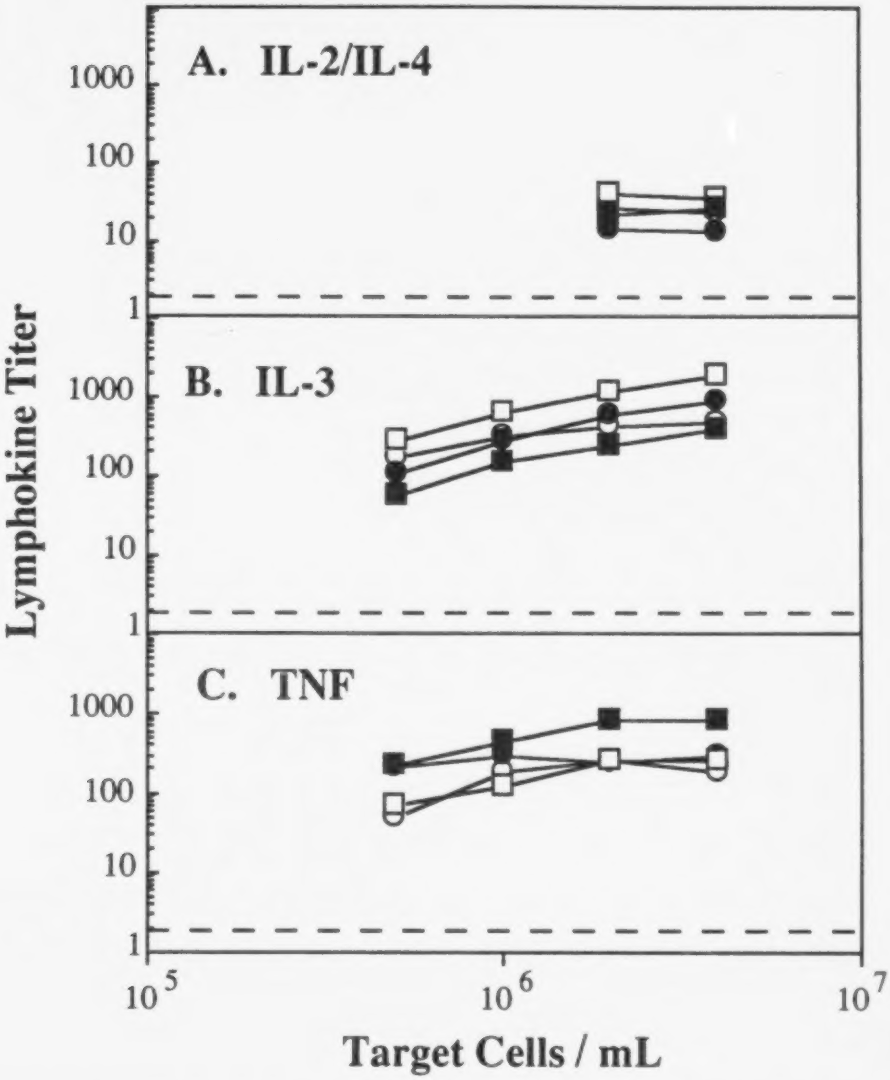

FIGURE III-3. ***Anti-donor lymphokine production from tolerant animals.*** Activated B6-specific T lymphocytes from tolerant spleen (□), tolerant lymph node (○), control spleen (■), and control lymph node (●) were assessed for (**A**) IL-2, (**B**) IL-3 and (**C**) TNF production in response to donor Class I MHC antigen-bearing target cells (EL-4, H-2 ^{b}). In vitro activated and subcultured lymphoid cells were incubated with UV-irradiated EL-4 cells for 6h. Supernatants were harvested and tested for lymphokine activity. The dashed line indicates activity from activated T cells cultured without antigen (medium alone).

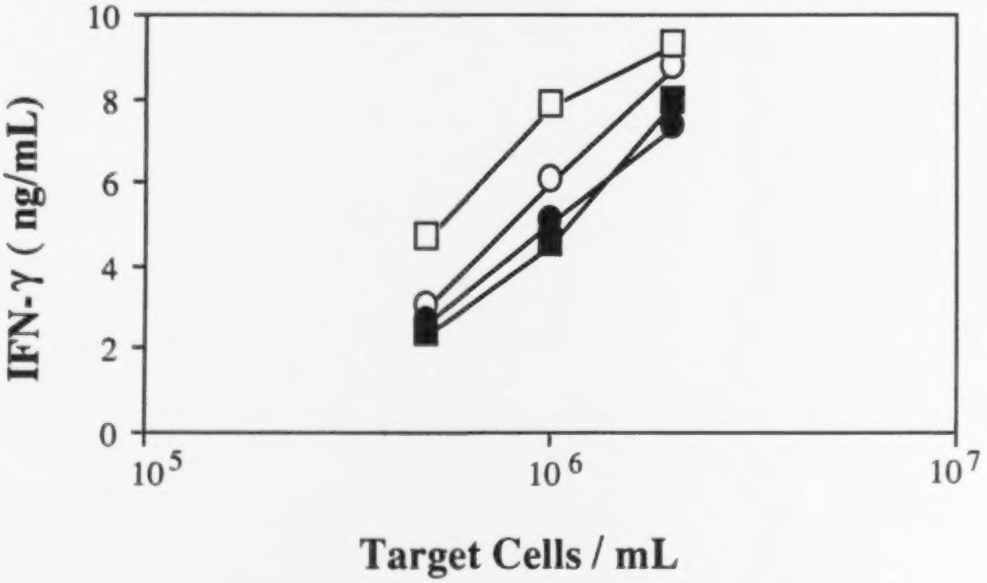

FIGURE III-4. ***Primed lymphoid cells from tolerant animals secrete IFN- γ in response to donor Class I MHC antigen (EL-4, H-2^b).*** In vitro primed LN (○●) and spleen cells (□■) from tolerant (open figures) and control (closed figures) animals were incubated with UV-irradiated EL-4 cells for 6h. Quantities of IFN- γ in culture supernatants were determined from a standard curve in a ELISA assay. Supernatants from cultures of responders in the absence of antigen contained amounts of IFN- γ below the lower limit of detectability of this assay (0.2 ng/mL).

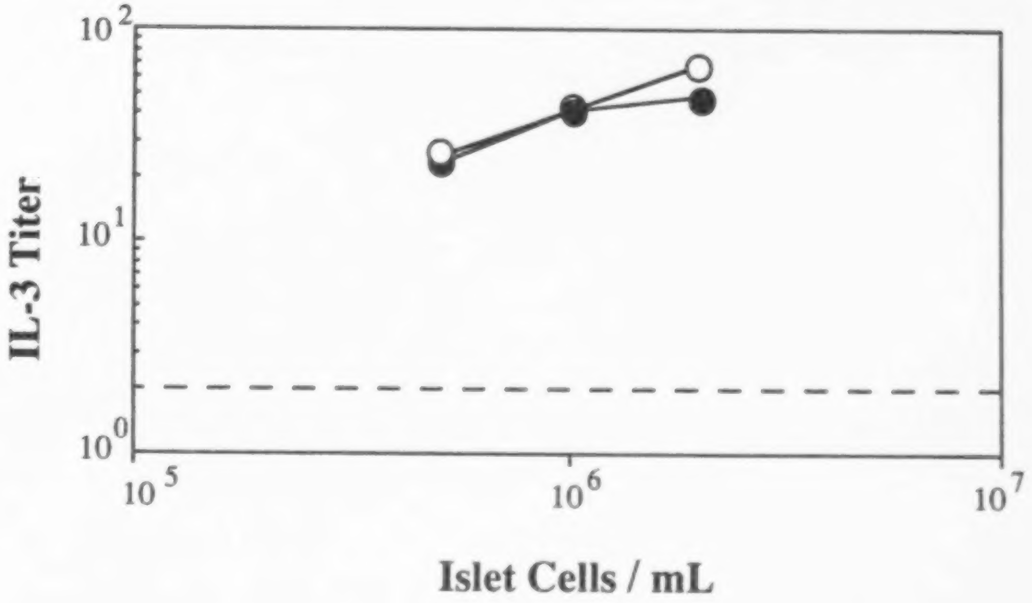

FIGURE III-5. ***In vitro activated lymphoid cells derived from tolerant animals are reactive to donor islet antigen.*** LN cells from tolerant (○) and control mice (●) were activated in vitro and triggered to produce IL-3 in response to UV-irradiated B6 islet cells for 6h. Values represent IL-3 activity in culture supernatants. The dashed line represents the level of IL-3 activity from activated T cells cultured in media alone (titer ≤ 2).

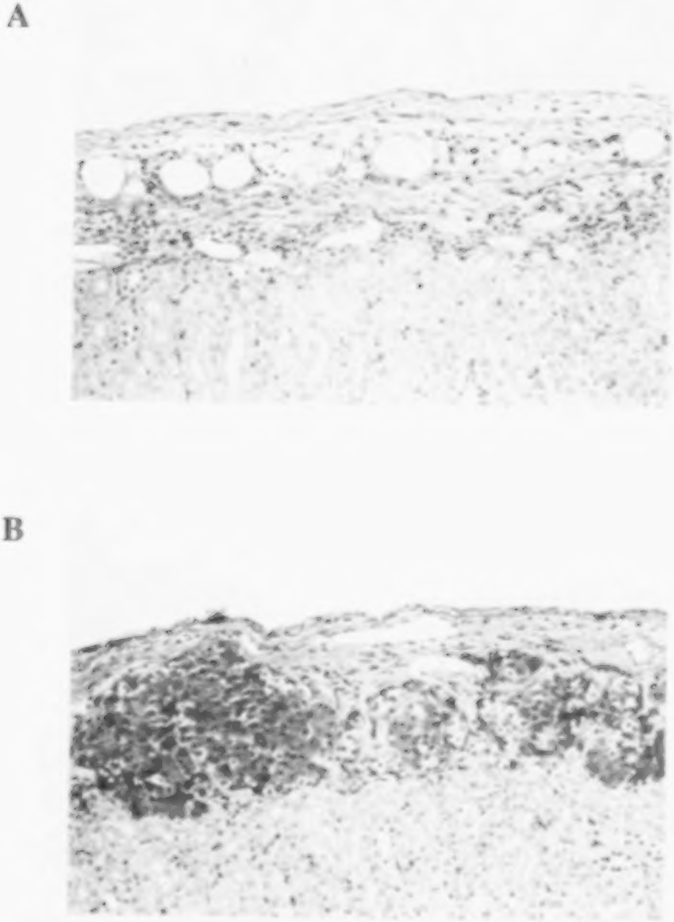

FIGURE III-6. *In vitro primed T cells from tolerant animals can mediate donor-specific islet graft destruction in vivo.* One million *in vitro* primed (BALB/c anti-B6) spleen cells from tolerant animals were placed adjacent to a B6 (**A**) or BALB/c (**B**) sentinel islet graft residing in a normal BALB/c recipient. Note the complete destruction of the B6 islet graft with remaining mononuclear cells and scar tissue in contrast to the well-granulated syngeneic islet tissue devoid of mononuclear cell infiltrate (Aldehyde-fuchsin, Original magnification x100).

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IV

LACK OF T CELL TOLERANCE TO EXTRATHYMIC ALLOANTIGEN

The deletion of autoreactive T cells that encounter self MHC antigens in the thymus appears to be a major mechanism responsible for the generation of self tolerance (1-3). Since it is improbable that all self antigens are expressed in the thymus, peripheral mechanisms of tolerance have been invoked to explain the lack of T cell reactivity to self MHC antigens expressed exclusively on extrathymic tissues. Transgenic models, in which antigen expression is targeted to specific extrathymic tissues, have been used extensively to investigate this issue. Such ectopic antigen expression on tissue parenchymal cells is thought to be tolerogenic (4), though the mechanism of tolerance remains controversial. T cell receptor occupancy (signal 1) in the absence of the second costimulatory (CoS) signal necessary for T cell activation has been shown to result in T cell clonal inactivation/anergy *in vitro* (5) and has been proposed to occur in some transgenic models of extrathymic tolerance (6-8). However, recent findings of low level expression of the transgene product in the thymus of MHC Class I transgenic mice complicates the interpretation of these studies (9, 10). Other mechanisms, such as deletion (11, 12) and the down-regulation of T cell receptors and/or CD8 molecules (13) have also been implicated in the induction of tolerance to extrathymic tissues. Conversely, there is also evidence to suggest that it may be unnecessary to invoke a mechanism for tolerance to peripheral self antigens (14). In this study, we addressed the issue of peripheral tolerance induction using a transplantation model.

The removal of APCs from tissue prior to transplantation reduces allograft immunogenicity such that the graft no longer activates host T cells directly (15). Such grafts provide 'signal 1' (T cell receptor occupancy) without the second CoS signal required for T cell activation. The evidence supporting this concept is the finding that APC-depleted allografts survive and function indefinitely in non-immunosuppressed allogeneic recipients (15). Such grafts acutely reject following host immunization with donor-type APCs early after grafting, indicating that the graft expresses recognizable alloantigen (15). APC-depleted allografts, therefore, are a useful tool for examining whether 'signal 1' antigen presentation, on the surface of cells incapable of supplying the second signal, leads to tolerance, specifically T cell clonal anergy, *in vivo*. We determined whether newly developing T cells were activated, tolerized or indifferent to antigen expressed on APC-depleted allografts residing in the periphery of severe-combined-immune-deficient (scid) mice. In this model system, physiological levels of cell surface antigens are expressed and intrathymic expression of the native alloantigen is precluded. Our results indicated that developing T cells were neither activated nor tolerized by alloantigens expressed on extrathymic parenchymal cells.

MATERIALS AND METHODS

Animals. Male BALB/c (H-2^d), C57BL/6 (B6, H-2^b) and CBA/J (CBA, H-2^k) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Severe-combined-immune-deficient C.B-17*scid/scid* (scid, H-2^d) mice were generously provided by L. Shultz and bred at the Barbara Davis Center animal facility.

Cell lines. Tumor cell lines, EL-4, a C57BL/6N (H-2^b) lymphoma and R1.1, derived from a C58/J (H-2^k) thymoma, were maintained by serial passage in Dulbecco's modified Eagle's minimal essential medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum.

Islet preparation and transplantation. Pancreatic islets, isolated from B6 donors by collagenase digestion (16) and Ficoll purification (17), were cultured in an atmosphere of 95% O_2 and 5% CO_2 as previously described (16). This pretransplant culture is thought to eliminate tissue immunogenicity by the depletion of resident antigen presenting cells (APC) (15). Six week old scid mice were rapidly rendered diabetic (consecutive blood glucose readings $\geq$ 20 mM) with one intraperitoneal injection of 225 mg/kg streptozotocin (Calbiochem, La Jolla, CA). Four hundred and fifty high O_2 cultured B6 islets were implanted to the renal subcapsular space of diabetic animals (16). Allograft function was assessed by monitoring blood glucose values weekly with an Exactech® blood glucose meter (MediSense, Inc. Cambridge, MA). Graft rejection was defined as the first of consecutive blood glucose values greater than 2SD above the normal range.

Bone marrow reconstitution of C.B-17*scid* mice. Bone marrow cells, obtained from femurs and tibias of BALB/c or B6 mice, were depleted of T cells with anti-thy 1.2 HO-13-4 culture supernatant (18) and complement (Low-Tox®-M rabbit complement, Cedarlane Laboratories, Hornby,Canada). T cell-depleted BALB/c bone marrow cells (5×10^6 cells) were injected into the tail vein of scid mice 2-3 weeks after islet grafting. Similarly, some mice received one injection of BALB/c, combined with

B6, T-depleted bone marrow in a 1:3 ratio (5×10^6 BALB/c plus 15×10^6 B6 cells).

Assessment of tolerance. Ten weeks after bone marrow grafting, mice were immunized with 10^6 B6 spleen cells as a source of donor-type APCs. Such spleen cells were depleted of T cells as described above. Blood glucose was monitored three times/week following host immunization. The maintenance of normoglycemia ≥ 3 weeks following APC challenge was indicative of tolerance induction.

Flow cytometry. Bone marrow cells, before and after T cell depletion, were directly labelled with FITC-conjugated rat anti-mouse CD4 (YTS 191.1, Caltag Laboratories, San Francisco, CA), FITC-rat anti-mouse CD8 (53-6.7, Becton Dickinson, Mountain View, CA), FITC hamster anti-mouse CD3ε (145-2C11; Pharmingen, San Diego, CA) and FITC-rat anti-mouse Ly-5 (B220, RA3-6B2; Pharmingen) mAb. Peripheral blood lymphocytes, isolated on Lympholyte M Ficoll gradients (Cedarlane Laboratories), were directly labelled with FITC-conjugated mAb to CD4, CD8 and goat anti-mouse Ig (IgG+IgM, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) as markers for T and B cells, respectively. Frequency determinations were calculated from single-parameter fluorescence histograms on an EPICS C® flow cytometer (Coulter Electronics, Hialeah, FL).

Mixed leukocyte cultures (MLC). The mixed leukocyte reaction was established by mixing 2×10^5 LN cells from BALB/c or reconstituted scid mice with 2000R-treated B6 or CBA splenic stimulator cells (3×10^5) in a total of 0.2 mL cultures in 96-well flat-bottom plates (Linbro). Cells were cultured in Eagle's Minimal Essential Medium (Gibco) supplemented with 10% fetal calf serum, 10^{-5}M 2-mercaptoethanol, and antibiotics and incubated at 37°C in 10% CO_2 in air. Proliferative responses were determined by pulsing cultures with 1.25 µCi [^{3}H]-thymidine for 6h on days 3, 4 and 5 of primary culture. For CTL assays, primary MLC were established in 24-well plates (Falcon) with $2x10^6$ LN cells as responders and $3x10^6$ 2000R-treated splenic stimulator cells.

^{51}Cr-release assay. Varying numbers of effector T cells, harvested on day 5 of MLC, were incubated with 10^4 ^{51}Cr-labelled tumor target cells for 4h at 37°C in 10% CO_2. Supernatants were harvested and ^{51}Cr release was detected on a gamma counter. Cytotoxic activity is expressed as $\log_{10}$ cytotoxic units (CU) per culture as previously described (19).

Histological examination of grafted tissues. Graft-bearing kidneys were removed and fixed in 10% formal saline. Paraffin sections were stained with aldehyde fuchsin (AF) to stain insulin-containing granules. Tissue sections were examined to determine the degree of tissue damage and mononuclear cell infiltration of the graft.

Statistical analysis. The Fisher's exact was used for comparisons between groups. The student t test was used to determine the significance between percentages of peripheral blood lymphocytes detected in BALB/c animals and bone marrow reconstituted scid mice.

RESULTS

Although C.B-17*scid* mice, Igh congenic to BALB/c, are devoid of mature T and B lymphocytes, immunocompetence can be restored by the adoptive transfer of hematopoietic stem cells (20, 21). Reconstitution of scid mice with 5×10^6 BALB/c T cell-depleted bone marrow cells established immunocompetence within 8-10 weeks. This was demonstrated by phenotypic analysis of peripheral blood lymphocytes; $CD4^+$ and $CD8^+$ T cells and Ig^+ B cells were detected in proportions equivalent to control BALB/c animals (Table IV-1). *In vitro* reactivity to donor-type (B6) and third-party (CBA) ($H\text{-}2^k$) stimulators in MLC (Figure IV-1) and CTL (Figure IV-2) assays was also comparable to BALB/c controls. In addition, in preliminary experiments, B6 thyroid allografts implanted into scid mice, 8 weeks after bone marrow reconstitution, were rapidly rejected while being uniformly accepted in unmanipulated scid mice (Table IV-2). These results demonstrated the immunocompetence of bone marrow reconstituted scid mice.

Transplantation of APC-depleted (high O_2 cultured) B6 islet allografts reversed streptozotocin-induced diabetes indefinitely (>100 days) in immuno-incompetent scid mice (Table IV-3, Group I). Scid mice bearing cultured B6 islet allografts which were reconstituted with 5×10^6 BALB/c T cell-depleted bone marrow cells 2-3 weeks following islet transplantation also remained normoglycemic for ≥ 10 weeks post-bone marrow grafting (Table IV-3, Groups II and III). At this time, the peripheral pool of T and B lymphocytes in bone-marrow reconstituted animals was normal, relative to unmanipulated BALB/c control animals. Thus, donor-specific T cells in reconstituted animals were not activated despite maturing in the presence of the B6 islet allograft residing in the periphery.

At ten weeks post bone-marrow grafting, we determined whether such T cells were tolerant to the B6 graft by immunizing the mice with 10^6

B6 spleen cells, as a source of APCs. This donor APC challenge resulted in the uniform rejection of the established islet allografts within 7-9 days after immunization (Group III), indicating that tolerance was not induced. Reconstituted mice, which were not challenged, remained normoglycemic for ≥100 days (Group II), indicating that appropriate host immunization was necessary for graft rejection. Further, host immunization with donor APCs, *per se*, did not lead to islet graft rejection in unreconstituted scid mice (Group IV) as animals in this group were normoglycemic for an additional 30 days after challenge. This result indicated that the rejection response was dependent on BALB/c T cells and not due to host-derived lymphocytes in potentially 'leaky' scid mice (22, 23). Therefore, T cells specific for B6 alloantigens were neither activated (Group III) nor tolerized (Group II), despite maturing in the presence of an established peripheral B6 islet graft.

At the conclusion of this study, graft-bearing kidneys were removed from all mice normoglycemic for more than 100 days. Nephrectomies led to a rapid rise in blood glucose values (hyperglycemia), demonstrating that the euglycemia observed in these animals was graft-dependent. Histological sections from scid mice reconstituted with BALB/c bone marrow, but not challenged, revealed intact islets full of insulin-containing granules and free of lymphocytic infiltration (Figure IV-3A). Islet allografts from unreconstituted scid mice, whether or not the animals were immunized with donor APCs, had a similar histological appearance. In contrast, grafts from bone marrow reconstituted and challenged scid mice were destroyed (Figure IV-3B); only residual mononuclear cell infiltrate was found at the graft site. These results indicated that tolerance was not induced to the peripheral APC-depleted allograft.

To demonstrate that a tolerant state could be established in scid mice bearing APC-depleted islet allografts, mice were reconstituted with a combination of BALB/c plus B6 T-depleted bone marrow cells. FACS analysis of peripheral blood demonstrated a state of chimerism in a proportion of these mice 10 weeks after reconstitution (data not shown). Then, following immunization with 10^6 donor spleen cells, these mice remained normoglycemic. Grafts harvested three weeks later were intact and free of graft-destructive inflammation. In proliferative and cytotoxic assays, lymph node cells from these animals did not respond to donor-type B6 stimulators but responded to third-party (CBA) stimulator cells at levels comparable to BALB/c control animals (data not shown). These results indicated that a deletional form of tolerance could be induced in scid mice under appropriate conditions.

DISCUSSION

The two-signal model for T cell activation (24), adapted from the Bretscher/Cohn model for B cell induction (25), proposed that two distinct signals, T cell receptor occupancy (signal 1) and costimulatory activity (signal 2) from a metabolically active stimulator (S^+) cell, were necessary for induction of the T cell. A corollary of this proposal was that the delivery of signal 1 alone was tolerogenic. Jenkins and Schwartz (26) demonstrated experimental support for this concept by showing that the presentation of signal 1 via chemically fixed APCs led to T cell clonal inactivation/anergy *in vitro*. The extent to which this process occurs *in vivo*, especially towards alloantigens, remains unclear. T cell clonal anergy resulting from the lack of CoS signals has been implicated in tolerance to transgenic Class I K^b (6) or Class II I-E (7) MHC alloantigens expressed on murine pancreatic β cells via the rat insulin promoter. Such transgenic studies have come into question due to potential low level expression of the transgenic product in the thymus (4). Importantly, thymus grafts from transgenic animals with putatively tissue-specific expression of K^b led to the induction of tolerance to K^b-bearing skin grafts in nontransgenic recipients (9, 10). These studies demonstrate that the thymus may play a role in the induction of tolerance in at least some transgenic models. It has also been demonstrated that Class II MHC I-E antigens must associate with endogenous superantigens to induce deletion or anergy (27, 28). These models, therefore, may not reflect conventional antigen presentation. For these reasons, the inference that T cell inactivation occurs as a result of signal 1 antigen presentation in the periphery, should be viewed with caution.

We set out to determine whether T cell interaction with an APC-depleted islet allograft - a model for signal 1 antigen presentation - would lead to the development of tolerance, specifically T cell clonal inactivation (anergy), in a newly developing immune system. It has been demonstrated that high O_2 cultured islet allografts are accepted long-term in adult immunocompetent recipients (29, 30). Such grafts, which are devoid of MHC Class II^+ cells (31, 32), do not elicit T cell activation, but can serve as a target for immune destruction when the host is immunized with donor-type APCs in the early post-transplant period (29, 30, 33). We utilized C.B-17*scid* mice as transplant recipients because allografts could be established before T cell development was reconstituted with lymphoid precursor cells. In this model, APC-depleted (cultured) islet allografts, bearing normal levels of cell surface Class I MHC alloantigens, would eliminate intrathymic expression of native alloantigens. Thus, the APC-depleted allograft becomes analogous to self antigen expressed exclusively on extrathymic nonlymphoid tissue, and, theoretically, should be perceived as 'self' by a newly developing immune system. Our results indicate that T cells were neither activated nor tolerized to peripheral antigens residing on tissue capable of providing 'signal 1' alone. As such, these findings are similar to a report by Ohashi *et al* (14) in which an LCMV glycoprotein, expressed exclusively on pancreatic β cells, did not induce autoimmunity. However, an immune response, leading to islet destruction and diabetes, occurred only when the host was immunized with the live virus. Taken together, these studies are consistent with the notion that T cells are indifferent, neither activated nor tolerized, when extrathymic alloantigens are presented on cells that cannot supply the second CoS signal. Rather, we would propose that tolerance induction may be a two signal process (34) and/or requires intrathymic antigen exposure (35). It should also be considered that the signal 1-induced anergic state can be prevented by exogenous IL-2 (5) or bystander APCs (36) *in vitro*, an event that is likely to occur *in vivo* by the local production of IL-2 and/or resident APC in the local microenvironment. In addition, it is unclear whether significant proportions of naive graft-reactive T cells directly circulate through tissues and encounter the graft. If such potentially reactive T cells do not extensively circulate through peripheral tissues, then inactivation of these cells via antigen exposure appears unlikely in normal self-tolerance induction.

Although studies from our laboratory (30, 33, 37) and others (38, 39) have indicated that an APC-depleted allograft *can* induce a state of donor-specific tolerance in adult animals, this form of tolerance does not appear to be due to a passive (deletion/inactivation) mechanism. Lymphoid populations from tolerant animals respond to donor antigen in a variety of *in vitro* assays and, *in vitro* primed T cells from tolerant animals have the capacity to destroy donor-type allografts *in vivo* (40). Thus, an active, regulatory mechanism appears to be involved in tolerance induction to APC-depleted allografts in adult recipients (33, 39, 40). It is intriguing that active mechanisms may also play a role in tolerance developing to self components during ontogeny (41-43). Since tolerance to APC-depleted grafts in adult animals develops slowly over time, the majority of animals becoming tolerant >100 days post-transplant (30,33,38) it will be interesting to see whether such a tolerant state develops to islet allografts in bone marrow reconstituted animals.

In conclusion, we have found that tolerance was not induced to APC-depleted tissue residing in the periphery during the maturation of the immune system. Signal 1 antigen presentation in the periphery, therefore, does not apparently lead to the development of T cell clonal anergy *in vivo*, consistent with the results of Ohashi *et al* (14). Thus, it may not always be necessary to invoke peripheral tolerance mechanisms to account for the lack of reactivity to self antigens unique to extrathymic tissues.

TABLE IV-1. FACS analysis of peripheral blood lymphocyte populations in naive BALB/c, C.B-17scid, and scid mice reconstituted with BALB/c bone marrow.

Group	**n**	**Peripheral Blood Lymphocytes (Mean ± SD)**		
		CD4+	**CD8+**	**IgG / IgM+**
BALB/c	8	47.1 ± 9.9[a]	13.2 ± 1.6[b]	23.8 ± 7.4[c]
BALB/c-->scid	14	49.0 ±13.5[a]	10.9 ± 2.0[b]	16.1 ± 9.6[c]
scid	7	6.0 ± 3.5 *[d]	0.5 ± 0.6[d]	0.6 ± 0.4[d]

C.B-17*scid* mice were reconstituted with 5x10^6 T cell-depleted BALB/c bone marrow cells (BALB/c--->scid). Eight weeks after reconstitution, peripheral blood lymphocytes were directly labeled with FITC-conjugated rat anti-mouse CD4 and CD8 mAb and FITC-conjugated goat anti-mouse IgG+IgM, as markers of T and B cells, respectively. Peripheral blood lymphocytes from naive BALB/c and unmanipulated scid mice are included as controls.

*Small proportions of very low intensity CD4$^+$ T cells were detected.

[a] NS (p = 0.740)

[b] p = 0.010

[c] NS (p = 0.064)

a, b, c versus *d*: $p < 0.0001$

TABLE IV-2. C.B-17*scid* mice are capable of rejecting B6 (H-2^b) thyroid allografts eight weeks after reconstitution with BALB/c (H-2^d) bone marrow cells.

Group	Bone marrow Reconstitution	B6 Thyroid Allograft Survival
I	---	2 / 2 [a]
II	+	0 / 6

Untreated B6 thyroids were implanted beneath the kidney capsule of allogeneic C.B-17*scid* which were either immuno-incompetent or reconstituted with $5x10^6$ T cell-depleted BALB/c bone marrow cells eight weeks earlier. Graft function was assessed, thirty days after grafting, by the uptake of ^{125}I (≥ 5 times the background cpms of the nongrafted control kidney) and was confirmed by histological examination.

[a] Group I vs II: $p = 0.036$

TABLE IV-3. Survival of APC-depleted C57BL/6 ($H\text{-}2^b$) islet allografts in C.B-17*scid* ($H\text{-}2^d$) mice following maturation of the immune system.

Group	BALB/c bone marrow	B6 APC Challenge	Islet Graft Survival (days) Pre-challenge	Post-challenge
I	--	--	6 / 6 (>100)	ND
II	+	--	7 / 7 (>100)	ND
III	+	+	6 / 6^a (70)	0 / $6^{a,b}$ (7,7,7,8,8,9)
IV	--	+	5 / 5 (70)	5 / 5^b (>30)

Diabetic scid mice were grafted with cultured (APC-depleted) B6 islet allografts and, after 2-3 weeks, were reconstituted with T-depleted BALB/c bone marrow cells. Ten weeks (day 70) following bone marrow grafting, animals were immunized with 10^6 T-depleted B6 spleen cells as a source of APCs. Graft survival was assessed by normoglycemia and confirmed by histological examination.

[a,b] $p = 0.0022$

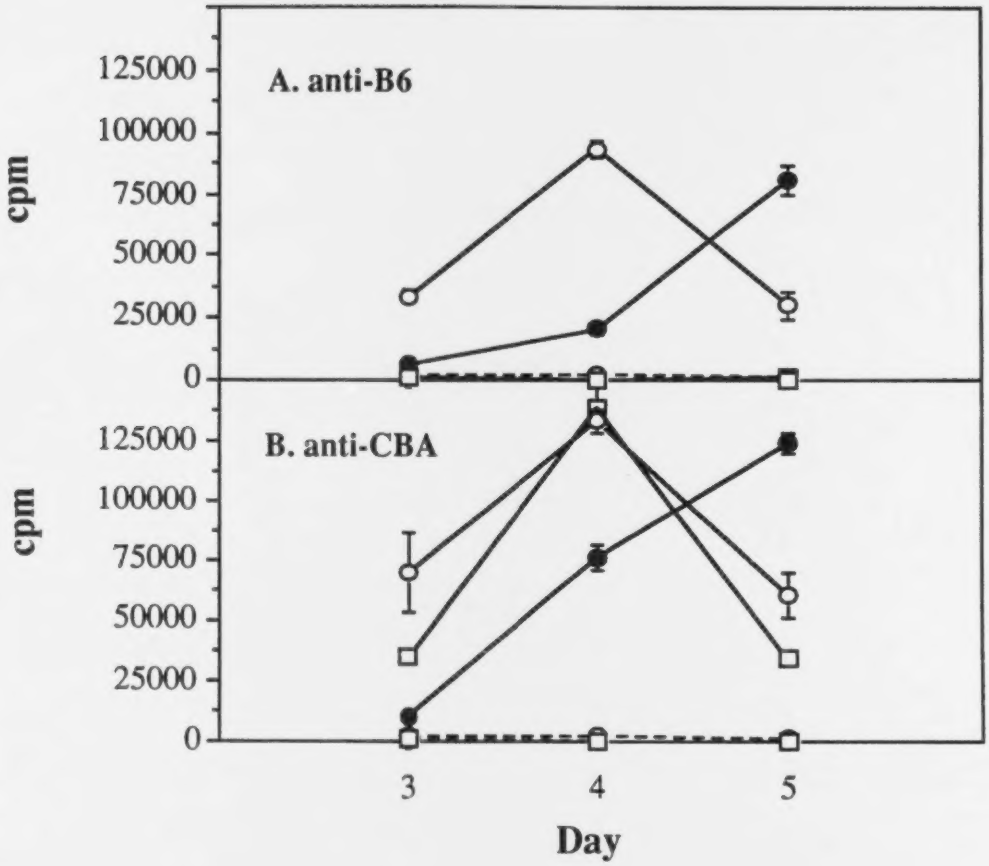


FIGURE IV-1. ***Proliferative responses of LN cell populations from BALB/c and bone marrow reconstituted C.B-17 scid mice bearing B6 islet allografts.*** LN cells from naive BALB/c mice (○) or scid mice grafted with cultured B6 islets and then reconstituted with T cell-depleted BALB/c (●) or BALB/c + B6 (□) bone marrow, were incubated with (——) or without (----) 3 x 10^5 irradiated B6 (**A**) or CBA (**B**) spleen cell stimulators. On days 3, 4 and 5, cultures were pulsed with [^{3}H]-thymidine and harvested 6h later.

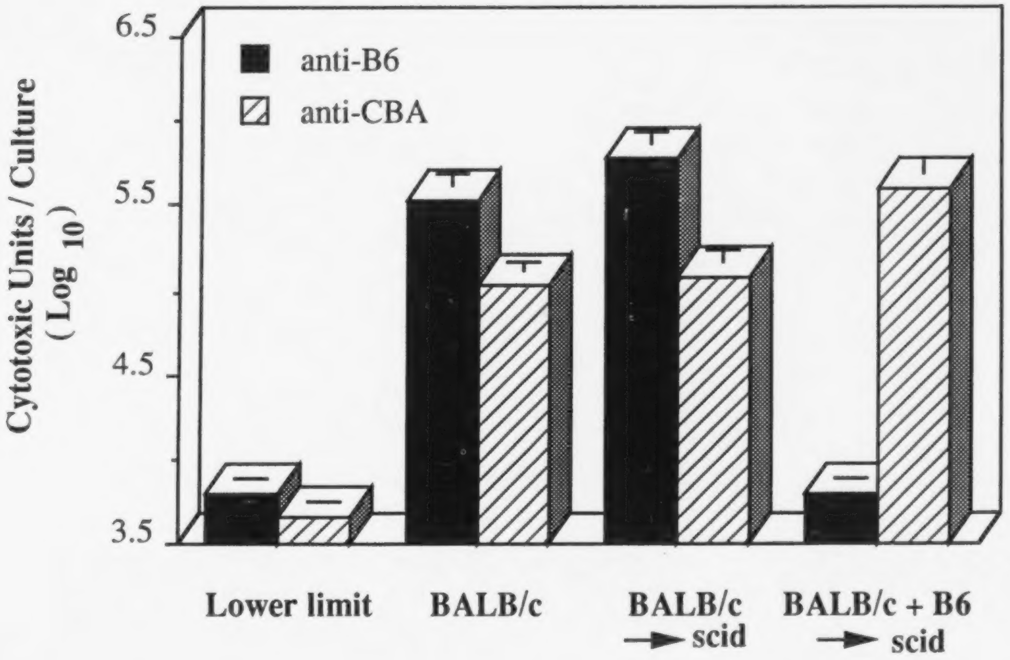
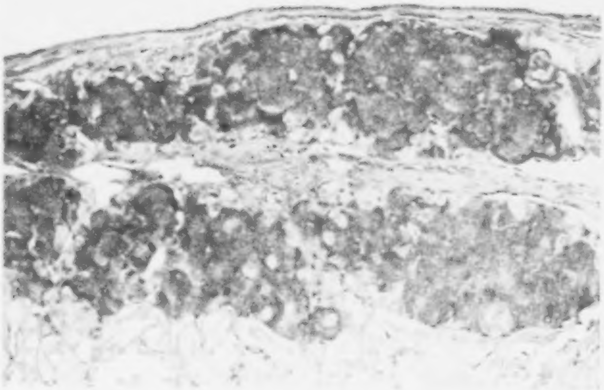


FIGURE IV-2. ***Cytotoxic responses of LN cells from naive BALB/c or bone marrow reconstituted scid mice bearing cultured B6 islet allografts.*** LN cells, activated with B6 or CBA stimulator cells, were harvested on day 5 and assessed for cytotoxicity against donor (EL-4, H-2^b) or third-party (R1.1, H-2^k) Class I antigen bearing targets in a 4h ^{51}Cr release assay.

A



B

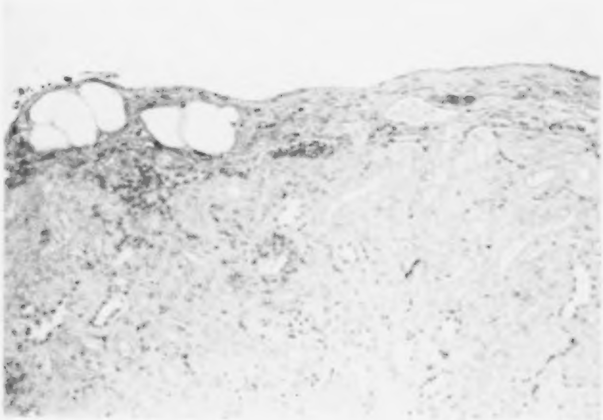


FIGURE IV-3. *APC-depleted B6 islet allografts in C.B-17 scid mice reconstituted with BALB/c bone marrow*. **A.** An islet allograft residing in a scid mouse 100 days after bone marrow reconstitution. AF staining reveals insulin granules in intact islets. Note the lack of lymphocytic infiltration. **B.** An islet allograft in a bone marrow reconstituted scid mouse, challenged with 10^6 donor-type B6 spleen cells 70 days after bone marrow grafting. Note the complete destruction of islet tissue and residual mononuclear cell infiltration. (Original magnification x 100).

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V

DISCUSSION

The ability to deplete pancreatic islets of associated APCs and obtain indefinite allograft survival deserves attention in the move toward clinical trials of islet transplantation for the treatment of insulin dependent diabetes mellitus. Such tissue pretreatment might allow islet allografting with minimal or no recipient immunosuppression. The observation that such tissue treatment results in the induction of donor-specific tolerance makes this an even more advantageous option. Clinical application will, of course, require additional trials in other animal models and, ultimately, in man. The APC-depleted islet allograft is also a useful tool for investigating basic mechanisms of tolerance induction in either the adult animal or in a newly developing immune system. Before any tolerance-inducing strategies can be applied clinically in areas of transplantation or autoimmune disease, understanding of the mechanisms involved is of critical importance.

Antigenicity versus immunogenicity

Pancreatic islets of Langerhans can be depleted of immunostimulatory cells by a period of high oxygen culture (1). Such APC-depleted islets, when grafted into an allogeneic host, can supply a source of antigen (signal 1) but cannot initiate T cell activation because they lack the capacity to deliver a second costimulatory signal required for T cell induction. The allograft, then, appears immunologically 'silent' in that host anti-donor T cells are neither activated nor tolerized in the early post-transplant period. Graft acceptance is not due to the loss of graft antigen during the period of high O_2 culture as the graft can be recognized and rejected when the host is immunized with a source of donor APCs. Further, several groups have demonstrated that increased MHC antigen expression on the surface of islet (2, 3) or thyroid (4) tissue does not increase its immunogenicity. Similar results with high O_2 cultured islets are presented in Appendix B. Pancreatic islets were incubated with IFN-γ during the last four days of high O_2 culture. MHC Class I antigen expression, as assessed by immunohistochemistry, was markedly enhanced. However, despite this hyper-expression of Class I MHC antigen, such grafts were not rejected in allogeneic recipients. Thus, the ability of high O_2 cultured islet allografts to function indefinitely in an immunocompetent allogeneic host, and their susceptibility to rejection following host immunization with a source of S^+ cells, indicates that MHC antigen alone is not a sufficient stimulus for allograft rejection. Further, these observations do not support the classical model of allogeneic reactivity which proposes that MHC antigen, *per se*, drives the immune response.

Long-term allograft acceptance versus tolerance

Long-term allograft acceptance is sometimes equated with tolerance induction. Without a clear definition of tolerance, allografts residing in an immunoprivileged site or an immuno-incompetent host could be classified as tolerant. A simple study illustrated the difference between long-term allograft acceptance and tolerance induction. When SZ-induced diabetic BALB/c mice were grafted with untreated B6 islets under the cover of anti-CD4 mAb or short-term treatment with mycophenolic mofetil (formally RS-61443), euglycemia was rapidly restored (5). Both immunosuppressive regimens led to indefinite islet allograft acceptance. However, only treatment with mycophenolic mofetil led to tolerance induction as assessed by maintenance of normoglycemia following active immunization of the recipients with donor spleen cells. Challenge with donor-type spleen cells resulted in islet allograft rejection in anti-CD4-treated recipients. Thus, long-term allograft acceptance does not necessarily mean that there has been a donor-specific change in the immune response of the recipient.

Experimental variables which influence graft stabilization

It has been demonstrated that APC-depleted allografts survive and function indefinitely in non-immunosuppressed recipients. Also, such grafts can serve as a target for immune destruction when the host is immunized with a source of APC in the early post-transplant period (the *metastable* graft). With time after transplantation, animals carrying cultured thyroid (6) or islet (7) allografts become progressively more resistant to graft rejection following immunization with donor APCs (*graft stabilization*). Variability in the proportions of animals bearing *stable* grafts has been observed. Such variability may be due to tissue (8) and/or strain (9) differences. For example, stabilization of cultured thyroid allografts in the BALB/c --> CBA strain combination was not observed at 100 days post-transplantation but was evident in 50% of animals 350 days after grafting (6). In contrast, when the kinetics of islet allograft stabilization in the BALB/c to CBA strain combination were examined, stabilization, as defined by the resistance to graft rejection by donor strain spleen cells, was observed in 100% of recipients challenged 120 days following transplantation (7). At the time of this study, 450 to 550 cultured islets were necessary to achieve normoglycemia in streptozotocin-induced diabetic recipients. With improvements in the quality of islet isolation, 350 cultured islets were capable of restoring euglycemia in diabetic hosts, however, the frequency of graft stabilization fell to 25% (Chapter II, Table II-1). Thus, the frequency of islet allograft stabilization was time-dependent and apparently influenced by the islet mass grafted. These parameters were systematically compared in the BALB/c --> CBA strain combination to determine factors which influence the state of islet allograft stabilization (Chapter II, Table II-1). The frequency of stabilization significantly increased with the time between transplantation and donor APC challenge as well as with a larger islet mass transplanted. Thus, these factors should be taken into consideration in further studies.

As with many immunological findings, another factor that may influence graft stabilization is the donor-recipient strain combination. The strain dependence of this phenomenon was demonstrated earlier in La Rosa's cultured thyroid studies (9) so it was important to demonstrate that islet allograft stabilization could be observed in greater than one strain combination. The B6 --> BALB/c strain combination was chosen to facilitate subsequent adoptive transfer experiments using the C.B-17*scid* (H-2^d) mouse. In this strain combination, a high percentage of animals resisted graft rejection following immunization with B6 spleen cells 120 days after grafting (Chapter II, Figure 1). A significant difference between graft survival at 30 versus 120 days post-transplant illustrated the time-dependence of graft stabilization. Thus, the phenomenon of islet allograft stabilization was demonstrated in a second strain combination.

With this characterization of the *stable* state, a key issue to be clarified in this thesis was: what is the mechanism responsible for graft stabilization? The two theoretical possibilities investigated were 1) graft adaptation to the host and 2) a change in the host to the graft (tolerance).

Adaptation of the islet allograft does not account for graft stabilization

Although previous work indicated that down-regulation of MHC antigen expression, *per se*, could not account for the initial acceptance of an APC-depleted allograft, it was possible that a change in graft antigen expression over time could account for the phenomenon of graft stabilization. Indeed, questions as to whether this phenomenon occurs often arise. In a previous study of thyroid graft stabilization, graft adaptation was excluded in an experiment in which the established *stable* allograft was removed from beneath the kidney capsule and retransplanted into naive recipients, syngeneic with the original recipient (6). A short period was allowed for re-engraftment and then the host was challenged with donor APCs. These grafts were rejected indicating that they still carried recognizable antigen. It can be argued that surgical trauma associated with re-transplantation may cause local inflammation and, possibly, upregulation of MHC antigens on the allograft which then makes the graft recognizable by T cells. Thus, it was important to demonstrate that graft adaptation alone could not account for the phenomenon of graft stabilization. This became particularly important in light of reports suggesting that down-regulation of MHC antigen expression may also play a role in culture-facilitated allograft acceptance (10-12) and resistance to subsequent donor APC challenge (10).

The exclusion of graft adaptation as a mechanism of islet graft stabilization was demonstrated by two different experiments. First, recipients bearing cultured islet allografts established for a long-term period (120 days) were able to protect secondary cultured B6 islet grafts carried in the animal for a short-term period (30 days), from donor-APC triggered rejection (Table II-2). Following immunization with donor APCs, age-matched control animals uniformly rejected B6 islet allografts residing in the host for the same short-term period. This experiment demonstrated that a host component was involved in the phenomenon of graft stabilization. Second, the long-term residence of cultured B6 islet allografts in C.B-17*scid* mice, independent of host immunocompetence, was not sufficient for graft stabilization (Table II-3). Together, these two experiments indicated that a loss or down-regulation of antigen on the graft over time was neither necessary nor sufficient for graft stabilization. Further, a host component involved in the process of graft stabilization was demonstrated by the ability to adoptively transfer protection of donor-type islet grafts and immunity to third-party islet grafts in scid mice (Table II-4). These studies indicate that graft stabilization is due to a donor-specific change in the host (tolerance) and not to a change in the antigenic composition of the graft (adaptation).

Mechanism of tolerance to APC-depleted grafts (active versus passive)

A corollary of two-signal models for lymphocyte induction was the proposition that delivery of signal 1 alone was tolerogenic (13, 14). The stimulator cell model of alloreactivity implied that an allograft depleted of S^+ cells delivered only signal 1 and, as such, cultured allografts become an excellent model of signal 1 antigen presentation. Previous reports on the mechanism of tolerance to cultured allografts implicated a role for an active mechanism in this process (6, 7, 15, 16). However, in recent years, tolerance induced to cultured allografts has been referred to as evidence for 'signal 1 anergy' (17-19). This has come with the renewed interest in the notion that delivery of signal 1, in the absence of CoS activity, leads to T cell inactivation or anergy. Since the Jenkins and Schwartz (20) demonstration that antigen presentation by chemically fixed APCs rendered T cell clones refractory to subsequent immunogenic stimuli, there has been numerous studies citing T cell anergy *in vivo*. Schwartz (21) has proposed that T cell anergy may explain self tolerance and, further, that there is potential for 'anergy-based' therapies in organ transplantation and autoimmune disease. For example, treating transplant recipients with drugs or antibodies that interfere with the expression of a proposed costimulator (B7/BB1) or its ligand CD28 on T cells may induce T cell anergy and transplantation tolerance (21). In support, of this idea, tolerance to xenogeneic pancreatic islet grafts was induced by blocking B7/BB1 molecules *in vivo* (22). Thus it was important to determine whether the tolerant state observed in animals bearing stable cultured allografts was due to a passive (deletion/inactivation) or active (suppressive, regulatory) mechanism.

Assessment of anti-donor reactivity in vitro

To determine whether the deletion or inactivation of donor-reactive cells occurred in tolerant animals, the ability of tolerant lymphoid populations to respond to donor antigen in vitro was compared to that of age-matched control (non-grafted) animals. Bulk MLC proliferative responses to donor (B6) stimulator cells indicated that both LN and spleen cell populations from tolerant animals were similar to controls (Figure III-1). In some tolerant animals, proliferative responses to donor antigen actually appeared primed as determined by an earlier peak response.

However, these proliferation assays measure responses of both CD4$^+$ and CD8$^+$ T cell subsets; previous experiments had demonstrated that primed CD8$^+$ T cells were both necessary and sufficient to trigger the rejection of established cultured allografts (23-25). Since pancreatic islets are MHC Class I$^+$, Class II$^-$ (26, 27), it was important to assess the anti-Class I (CD8-dependent) response of recipient T cells. Therefore, MHC Class I$^+$, Class II$^-$ target cells were used to measure the CD8$^+$ T cell response of tolerant animals. Donor-specific CTL precursor frequencies, assessed by limiting dilution analysis, and bulk cytotoxic responses to donor H-2^b antigen were similar to control responses. Because lymphokine production by the primed T cell plays an important role in allograft rejection (28), it was important to determine whether primed T cells from tolerant animals were capable of producing cytokines in response to donor antigen. The production of IL-2/IL-4, IL-3, IFN-γ and TNF, cytokines implicated in allograft destruction, were assessed. Figures III-3 and III-4 illustrate that *in vitro* primed T cells, derived from tolerant lymph node or spleen cell populations, produced cytokines in amounts similar to lymphoid populations from control animals. Therefore, relative to age-matched control animals, the *in vitro* bulk anti-donor Class I reactivity of tolerant animals was not impaired.

Assessment of anti-donor reactivity in vivo

A key element of the tolerant state is that the altered reactivity is specific to donor-type antigens. The adoptive transfer of spleen cells from tolerant animals to scid mice, bearing donor (B6) or third-party (CBA) sentinel grafts, (Table II-4) demonstrated that the tolerant state could be transferred in a donor-specific manner. To assess whether the alloreactivity of tolerant animals was altered specifically toward donor-type antigens of other tissues, BALB/c animals bearing stable B6 islet allografts were grafted with untreated donor-strain (B6) and third-party (CBA) thyroid lobes. In contrast to age-matched controls, approximately half of the tolerant animals accepted donor-type thyroids while simultaneously rejecting third-party strain grafts (Table II-5). This experiment demonstrated *in vivo* altered reactivity that was donor specific. Importantly, the rejection of secondary donor-type thyroid grafts in a proportion of animals did not induce the rejection of the primary islet allograft. This 'split tolerance', that is, acceptance of the primary islet allograft and rejection of secondary donor-type thyroid grafts, could, theoretically, be due to either: 1) adaptation of the primary islet graft or 2) tissue specificity of the tolerant state. The first possibility (graft adaptation) was shown not to be sufficient to account for the maintenance of the islet allograft in the two experiments previously described. Thus, the issue of 'tissue specific' tolerance was raised. This was a particularly important issue to resolve because, if there was a tissue-specific component to the tolerant state, did the tumor cells, used to assess *in vitro* reactivity, express the relevant tolerizing antigens?

Tissue-specificity of the tolerant state

The 'split tolerance' observed when secondary thyroid allografts were placed in animals bearing stable B6 islet allografts raised the issue of tissue-specific tolerance. T cell reactivity to tissue-specific peptides has been reported (29, 30) so it was possible that tolerance was generated in response to islet-specific peptides, *not* represented by the hematopoietic S^+ cells used as stimulators *in vitro*. It was also conceivable that tolerance in the islet allograft recipient was 'tissue-specific' with respect to differences in MHC Class I versus Class II antigen expression between islet and thyroid tissues. Whereas cultured islets express only Class I MHC antigen (31), uncultured thyroids express both Class I and Class II MHC antigen (27). In addition, thyroid tissue upregulates Class II antigen expression in the presence of IFN-γ whereas islet tissue does not (27, 32). Because cultured islets express only Class I MHC antigen, there is no reason to assume that tolerance would be generated to donor Class II MHC antigen. Thus, rejection of secondary thyroid allografts could be a response to Class II antigen alone. In support of this idea, BALB/c animals, depleted of $CD8^+$ T cells, were capable of completely destroying B6 thyroid allografts (Appendix C, Table C2). This result implies that $CD4^+$ T cells alone were capable of rejecting thyroid allografts and indicates that the protection of islet allografts is at the level of Class I MHC antigen recognition. This finding, however, does not disprove the idea of a tissue-specific peptide.

Lack of T cell specificity to an islet-derived peptide

The notion that T cells can recognize tissue-specific peptides came following the elucidation of the crystal structure of the human Class I MHC antigen, HLA-A2 (33). This led to a model of antigen presentation in which the TCR binds to MHC antigen plus a peptide fragment found in a peptide-binding groove, formed by the heavy chain $\alpha 1$ and $\alpha 2$ domains of the Class I MHC molecule (34). A similar hypothetical model for the physical interaction of the peptide with the MHC Class II molecule has been proposed (35). Such peptide-MHC binding is thought to be necessary for the expression of Class I MHC on the cell surface as the peptide stabilizes the Class I heavy chain - β2 microglobulin interaction (36). Also, self antigens have been shown to be constitutively processed and presented by APCs (37). There appears, however, to be considerable heterogeneity in the recognition requirements of alloreactive T cells *in vitro*. Experimental evidence has indicated that some alloreactive T cells are exclusively specific for endogenous peptides in association with MHC and others can recognize allogeneic MHC without any apparent requirement for peptide (30).

The notion that T cells may recognize allogeneic MHC plus an associated peptide would imply that graft-specific peptides could be presented by allo-MHC molecules and, therefore, that alloimmunity would have a tissue-specific component. However, there is little evidence for tissue-specific alloimmunity. For example, hematopoietic cells injected neonatally, as in the original Billingham, Brent and Medawar model (38), can induce tolerance to subsequent donor-type skin and organ grafts. Data shown in Table 11-5, along with a previous study (39), demonstrates that recipients of stable islet allografts accept a significant proportion of donor-type thyroid allografts. However, it was still conceivable that T cells specific for self MHC plus a donor islet-specific peptide were responsible for the tolerant state *in vivo* and the apparent anti-donor reactivity observed *in vitro*.

Tolerance generated in response to an islet-specific peptide may not be reflected by *in vitro* stimulation with donor-type hematopoietic cells, that is, the deletion/inactivation of T cells specific for islet-derived peptides would be masked by the overwhelming response of T cells reactive to peptides presented by spleen cell stimulators. To resolve this issue, the ability of *in vitro* primed T cells from tolerant animals to respond to islet antigen was examined. *In vitro* activated lymphocytes from tolerant animals were able to produce IL-3 in response to donor (B6) islet cells as antigen (Figure III-5). IL-3 was the lymphokine chosen for this experiment because the bioassay for IL-3 production was both sensitive to low antigen concentration and reproducible. A defect in the IL-2 pathway has been demonstrated in the Schwartz model of anergy (40) and in allograft tolerance developing in response to pretransplant blood transfusion (41). It is certainly conceivable that production of other lymphokines, especially IL-2, could be selectively blocked in response to islet cell antigen. It is also possible that T cells with high affinity for alloantigen are deleted or inactivated in tolerant animals so that only cells of lower affinity are left. The requirements for T cell activation *in vitro* may be less stringent than *in vivo* such that cells of lower affinity can respond *in vitro* when 'help' is provided. Thus, it was important to determine whether the *in vitro* activated T cells derived from tolerant animals had the capacity to destroy B6 islet allografts *in vivo*. When *in vitro* activated T cells from tolerant animals were placed adjacent to established sentinel grafts, the destruction of donor-type islet grafts, without effect on syngeneic BALB/c grafts, indicated that these cells were fully capable of mediating allograft rejection *in vivo* (Table III-2, Figure III-6). These results indicated that tolerant animals retain potentially reactive donor-specific T cells.

It is possible that tolerant cells are defective in their capacity to home to the graft due to changes in their cell surface adhesion molecules (42). This does not appear to be the case as both donor-type and third-party islet grafts in scid mice were rejected following peripheral administration of primed T cells (Appendix C Table C-2). Taken together, these results indicate that tolerant animals bear T cells which, once activated, have the functional capacity to mediate islet allograft destruction *in vivo*.

In summary, T cells from adult animals bearing APC-depleted allografts are capable of proliferating and generating cytotoxic activity in response to donor antigen *in vitro*. Such primed T cells secrete lymphokines in response to donor MHC and islet antigen *in vitro* and are capable of destroying donor-type islet grafts *in vivo*. Thus, the tolerance that develops in response to APC-depleted allografts is not due to the deletion/inactivation of donor-reactive T cells. This evidence does not support the notion that 'signal 1' antigen presentation leads to the inactivation (anergy) of donor-reactive T cells in adult animals. Rather, these results would be indicative of an active, regulatory form of tolerance.

Does 'signal 1' antigen presentation lead to the inactivation of newly developing T cells?

The tolerant state in the adult animal is not due to 'signal 1'-induced anergy. However, it is possible that signal 1 antigen presentation in the absence of signal 2 could lead to T cell inactivation during maturation of the immune system. Transgenic studies implicating signal 1 antigen presentation in T cell inactivation are models of developmental tolerance in which the test antigen is present during the ontogeny of the immune system. It was, therefore, necessary to determine whether recent thymic emigrants react differently to 'signal 1' antigen presentation than mature T cells in the adult? The recent barrage of reports implicating T cell clonal anergy as the mechanism of tolerance induction to peripheral self antigens led to the use of the APC-depleted graft to examine this possibility in a newly developing immune system. This model eliminated several problems associated with transgenic studies. For example, factors such as the timing of expression of the transgenic product or the level of transgene antigen expression on the cell surface may affect the immune response to the antigen (43). In addition, expression of the transgenic product in the thymus (44-46) complicates these studies. Thus, in transgenic models of peripheral tolerance induction, care must be taken to ensure that physiological levels of antigen are expressed on the surface of peripheral cells only. We utilized APC-depleted grafts to preclude the expression of native alloantigens in the thymus and the C.B-17*scid* mouse was used so that the allograft could be established prior to maturation of the immune system. When SZ-induced diabetic scid mice were grafted with cultured B6 islet allografts, blood glucose values returned to normal within a few days. These allografts functioned indefinitely in scid recipients reconstituted with T cell-depleted BALB/c bone marrow two weeks after islet transplantation, indicating that cultured grafts were accepted despite T cell maturation. Such bone marrow grafting resulted in immune competence within 8-10 weeks. This immunocompetence of reconstituted scid mice was assessed by: 1) FACS analysis of peripheral blood lymphocytes (Table IV-1), 2) *in vitro* reactivity (Figures IV-1 and IV-2) and 3) *in vivo* rejection of thyroid allografts (Table IV-2). After bone marrow reconstitution, immunization with donor-type spleen cells triggered the acute rejection of the established islet allografts (Table IV-3). Thus, T cells maturing in the presence of a B6 allograft are neither activated nor tolerized. It is not known whether such T cells ever encountered the peripheral alloantigen prior to host immunization, however, if such an encounter took place, it did not result in T cell inactivation (anergy).

This immunologically 'silent' state is very similar to the transgenic model of Ohashi *et al* (47) in which the LCMV glycoprotein was expressed exclusively on pancreatic β cells. No immune response to the viral gene product was observed until the host was immunized with live LCMV. In both of these situations, the foreign antigen, expressed on cells which lack CoS activity, was apparently ignored by the immune system. Only when the antigen was presented in an appropriate immunogenic manner was an immune response made. The 'metastable' APC-depleted allograft in immunocompetent adult animals also fits into this category of immunologically silent tissues; such grafts survive in the host until immunization with donor APCs triggers an immune response. Similarly, Markmann *et al* (48) demonstrated that MHC Class II I-E expressing fetal pancreas allografts were not rejected in I-E$^-$ mice until the host was immunized with donor-type APCs. Zinkernagel (49) has proposed that similar situations arise during certain viral infections or during tumor growth. Rabies or papilloma viruses can effectively evade the immune system by infecting neuronal cells or keratinocytes, respectively. Since these cells lack APC function and the viruses do not rapidly cause cell death, viral replication can continue unnoticed for some time. Only once extensive replication has taken place, will cell death occur. Then viral antigens can be processed and presented to T cells for T cell activation. However, by this time, the viral infection is well underway. Similarly, certain carcinomas or sarcomas may express tumor associated antigens but they cannot induce an immune response because they are not on APCs. Again, only when the tumor growth gets large enough that cell death occurs and efficient processing and presentation of tumor antigens has taken place, will an immune response be generated. Based on these observations, it is proposed that signal 1 presentation *in vivo* is a null event, neither activating nor tolerizing the reactive T cell.

Problems with tolerance induction by 'signal 1' antigen presentation

The hypothesis that TCR occupancy (signal 1) in the absence of a second costimulatory signal leads to T cell clonal inactivation (anergy) appears unlikely for several reasons. First, the evidence supporting the induction of anergy *in vivo* is debatable; some experimental results are technically flawed. Miller's group (50) has proposed that anergy develops in response to MHC Class I K^b-bearing islets. Immunity to the transgenic antigen did not occur *in vivo* and K^b-specific T cell unresponsiveness was observed *in vitro*. Although great effort was made to ensure that the MHC Class I K^b transgene product was not expressed in the thymus (44), two groups have demonstrated that when the thymus from an MHC Class I K^b transgenic mouse was grafted to a naive recipient, tolerance to the transgenic product was induced (45, 46). Recipients of the transgenic thymuses were able to accept K^{b+} skin grafts and reject third-party allografts, indicating that the thymus itself was capable of inducing the tolerant state. This finding seriously compromises the interpretation of previous studies as models of peripheral tolerance. The low level expression of the transgene product in the thymus could cause deletion of high affinity K^b-reactive T cells, allowing cells of lower affinity to enter the periphery. Such low affinity T cells would not be able to mediate an immune response to the transgene product *in vivo*, however, they could respond to K^b antigen when exogenous IL-2 is added *in vitro*. This interpretation would be consistent with experimental findings (44, 51).

The experiments by Lo and colleagues (52) examine T cell responses in animals expressing the MHC Class II I-E antigen on pancreatic β cells or acinar tissue. These animals were functionally tolerant to the transgene product *in vivo* and I-E reactive T cells were unable to respond to *in vitro* stimulation with mAb specific for particular TCR V$_\beta$ determinants (53). Interestingly, both thymocytes and peripheral lymphocytes were unresponsive to such TCR V$_\beta$ region crosslinking with mAb (53). Again, this latter finding could be indicative of thymic involvement in the tolerant state. That the lack of *in vitro* reactivity could be due to something peculiar about the I-E transgene product, itself, was suggested by studies in which the MHC Class II I-A transgene product was exclusively expressed on peripheral tissues. In these studies, tolerance was generated *in vivo* and yet T cells reacted strongly to the I-A antigen *in vitro* (54-56). It is possible that the differences in these studies is related to the association of I-E with endogenous superantigens. The I-E reactivity of T cells bearing particular V$_\beta$ domains has been shown to be mediated by retrovirus-encoded SAg (57), that is, the I-E alloantigen must be presented to T cells in association with an endogenous SAg for it to induce anergy or deletion (57). Superantigens are known to bind outside the conventional MHC peptide groove (58), thus, one must be cautious in extrapolating tolerance mechanisms pertaining to models of SAg to models of alloantigens.

In most of the transgenic studies mentioned above, active mechanisms have not been ruled out. When experiments were done to distinguish passive versus active mechanism, the results were quite interesting. Lo *et al* (59) utilized a model in which I-E was expressed exclusively on pancreatic acinar cells via the elastase promoter. As in the

I-E transgenic model, spontaneous autoimmunity was not observed and priming mice with I-E$^+$ spleen cells did not induce an immune response, indicating the animals were functionally tolerant. *In vitro*, T cells bearing I-E associated V_β domains were also unresponsive to TCR crosslinking *in vitro*. However, in this model, the infusion of naive nontransgenic T cells did ***not*** break the tolerant state, as would have been expected if tolerance was due to a passive (deletion/inactivation) mechanism. Evidence that an active mechanism played a role in tolerance induction came from an experiment in which irradiation of the transgenic mice, followed by reconstitution with lymphocytes from naive animals, led to extensive lymphocytic infiltration of the acinar pancreas. Further, T cell depletion of the transgenic mice by adult thymectomy and mAb therapy, followed by the infusion of naive T cells also led to the destruction of the I-E$^+$ acinar tissue. This result indicated that the transgenic animal had T cells capable of mediating resistance to the immune destruction of the pancreas, that is, an active mechanism of tolerance was playing a role.

Despite problems with the interpretation of the above transgenic studies, the notion that 'signal 1' antigen presentation, in the absence of a second CoS signal, leads to T cell clonal anergy *in vivo* has been proposed to account for the self-nonself discriminatory feature of the immune system. There are theoretical problems with attempting to attribute tolerance induction, to peripheral self antigens or alloantigens, to solely passive mechanisms, particularly 'signal 1' induced anergy. Because T cells recognize peptide fragments bound to self MHC, the Lafferty/Cunningham (8, 14) two-signal model for T cell activation could not maintain the requirement for linked recognition of antigenic epitopes. This proposition, derived from the Bretscher/Cohn two-signal model (13), was key to explaining self-nonself discrimination. Without this proposition, potential problems arise. For example, suppose that a foreign viral antigen and a self antigen are expressed on the surface of the same extrathymic tissue. Naive T cells would not be able to differentiate between self or viral peptides on parenchymal (S^-) cells so if T cell interaction with self antigens on the surface of S^- cells induced clonal anergy, T cells with receptors for either antigen would be inactivated. T cells, tolerant to the viral peptide in association with self MHC antigen, would not be able to respond even when the viral antigen is processed and presented on the surface of an APC capable of supplying the second signal (S^+ cell). Because individuals would be unable to mount viral responses, it is very unlikely that this would occur. Similarly, if TCR occupancy is necessary to induce clonal deletion/inactivation, then each new T cell with anti-self specificity would have to circulate through every tissue to make contact with its specific antigen and be tolerized. This seems implausible as naive lymphocytes appear to circulate preferentially through lymphoid, rather than nonlymphoid, tissues (42).

Another problem with 'signal 1' anergy occurring *in vivo* is that *in vitro* studies indicate that the anergic state can be reversed with exogenous IL-2 (40, 60). Importantly, bystander APCs are also able to supply the second signal and prevent the anergic state (61). This reversibility implies that anergic cells could be rescued *in vivo* by the local production of IL-2 and/or resident APCs in the local microenvironment. Thus, T cells rendered anergic by interaction with self antigen + MHC in the absence of the second signal may be readily activated. Any local inflammatory response could potentially activate self-reactive T cells and autoimmunity would ensue. With this scenario, autoimmunity would be expected to be much more prevalent that it is. Thus, for several reasons, the idea that signal 1 antigen presentation alone leads to T cell clonal inactivation *in vivo* appears implausible. The results of the studies presented in this thesis indicate that S^- tissue, a model of signal 1 antigen presentation, does not lead to T cell clonal deletion/inactivation. Therefore, tolerance to APC-depleted islet allografts in adult animals is not due to a passive mechanism. Instead, an active, regulatory process is proposed.

Active mechanism of tolerance

In contrast to the Bretscher/Cohn model for lymphocyte induction (13), the two-signal model for T cell induction (8) appears to require some form of negative regulation other than the inactivation of T cells by signal 1 alone. Active mechanisms of tolerance have been implicated in tolerance developing to self components (62-65) and in many models of allograft tolerance (66). Additional evidence supporting a role for active, suppressive mechanisms of tolerance to self components comes from observations that immunodeficiencies or prolonged immunosuppressive therapies are associated with autoimmune disease (62, 67), indicating that T cells are necessary for normal self tolerance. If self tolerance is maintained by passive deletion/anergy mechanisms, then depleting T cells should have no effect on the development of autoimmune disease. Thus, these findings suggest that there is a mechanism protecting against destructive autoimmune responses, consistent with an active mechanism of tolerance.

The area of active 'suppressive' tolerance has faced years of frustration. During the 1970's, suppression, in the form of suppressor T cells, was thought to play a major role in allograft tolerance induction.

Although experiments demonstrating this suppressive phenomena were abundant, the search for characteristic markers of a suppressor T lymphocyte, such as the putative I-J molecule, was unsuccessful. In addition, T cell clones with long-term suppressive activity could not be generated and DNA sequencing techniques could not locate the elusive I-J locus (68). Therefore, support for such suppressive phenomena waned. It is possible that there is not a unique lineage of T cells bearing specific 'suppressor' markers functioning exclusively in suppressing immune responses. Rather, with the demonstration that the cytokine profile of murine T cell clones correlates with functional activity (69, 70), and with increasing knowledge of T cell regulation by cytokines (71), the suppressor phenomenology of the 1970's and '80's can be examined in a new light - immunoregulation.

Cytokines play an important role in the regulation of immune responses. This was demonstrated when certain $CD4^+$ T cell clones could be segregated into two functionally distinct groups, designated Th1 and Th2, based on the mutually exclusive pattern of cytokines they produced (69). Th1 clones produced cytokines such as IL-2, IFN-γ and TNF and were associated with inflammatory delayed-type hypersensitivity (DTH) responses. Th2 clones produced cytokines such as IL-4, IL-5 and IL-10. Such clones predominantly facilitated humoral responses. These observations provided an explanation for the earlier findings of Parish (72) who examined the cellular versus humoral responses of mice immunized with varying doses of antigen. He reported that as antibody titers increased in these animals, DTH responses simultaneously fell and vice versa. This switching between cell-mediated (DTH) and humoral classes of responses, a phenomenon referred to as immune deviation, has been recognized since the observation that immunizations which generated humoral responses led to reductions in DTH responses, normally observed after antigen exposure (73). These early studies have demonstrated that factors such as the nature and dose of antigen, previous exposure to antigen, or route of immunization can influence whether a cell-mediated or humoral response is induced (72, 74, 75).

In recent years, the ability of cytokines to regulate the outcome of an immune response has been well demonstrated in a model of murine parasitic infection with *Leishmania major* (71). Susceptibility to a progressive and fatal infection correlates with a Th2-like pattern of cytokine production and a humoral response to the parasite. In resistant strains, infections are characterized by a DTH response and a Th1-like cytokine profile. Thus, the outcome of the infection is not due to the presence or absence of an immune response, *per se*, but rather is related to the type of response as dictated by cytokine production. Importantly, perturbing the cytokine network can deviate the response from progressive to resistant or vice versa. For example, IL-4 (76) or antibodies to IFN-γ (77), given at the time of infection, will switch the DTH Th1-type response of resistant strains to the Th2-type response and the infection will be fatal. Conversely, when susceptible strains are treated with antibodies to IL-4 soon after infection, T cell cytokine secretion switches from the predominant Th2 pattern to a predominantly Th1-like response. Such mice then become resistant to the infection (78). Thus, the outcome of a particular immune response can be altered by the cytokines produced. Similar findings have been reported for other parasitic infections in mice (79) and leprosy infections in man (80). Differential cytokine profiles may also dictate whether an immune response to an allograft is driven towards rejection (a Th1-like response) or tolerance induction (a Th2-like response) (28, 81).

Immune deviation in allograft tolerance

Although still speculative, the idea that alterations in cytokine production may shift allograft immunity from a graft destructive response to a protective one is intriguing. It is possible that active forms of tolerance could be explained by altered T cell regulatory processes. The differential activation of Th2-like cells has been proposed to account for heart allograft tolerance induced by DSBT, anti-CD4 mAb therapy or short-term administration of CsA (81). In this study, analysis of cytokine transcripts from tolerated hearts revealed a reduction of IL-2/IFN-γ expression and enhanced expression of IL-4/IL-10 transcripts. Other models of allograft tolerance in neonatal (82, 83) or adult (41, 84, 85) animals may also be due to the differential regulation of cytokine producing T cells. Such a model would also explain the 'infectious' nature of allograft tolerance observed by Qin *et al* (86); cytokines produced by one cell may influence the subsequent cytokine production by another cell (87). Obviously, the role of cytokine interactions in immunity versus tolerance warrants further investigation.

Proposed mechanism of tolerance to APC-depleted allografts

Given that tolerance induction to APC-depleted allografts takes time to develop and is not due to T cell clonal deletion or inactivation, how might this form of tolerance be mediated? It is proposed that antigens (either MHC or non-MHC encoded) shed from an allograft can be processed and presented like any exogenous antigen. Uptake and processing of the exogenous antigen leads primarily to presentation by Class II MHC and, thus, activation of CD4$^+$ T cells (88). Such *indirect* T cells, however, would not be graft-specific as they would be restricted to graft antigen in the context of self (not donor) MHC molecules (Appendix A). CD4$^+$ T cells activated via the indirect pathway of antigen presentation could: 1) provide 'help' for graft destructive CD8$^+$ T cells, 2) 'help' B cells in the production of graft-specific antibody or 3) mediate a DTH response. It is conceivable that CD4$^+$ T cells, although not graft-reactive, could act as a source of 'help' for graft-specific cytotoxic CD8$^+$ T cells. A model of such T-T collaboration was initially proposed by Bach in 1976 (89). Although it has been demonstrated that production of lymphokines from helper T cells can activate bystander T cells of a different specificity *in vitro* (89-91), this type of T-T interaction does not appear to be very efficient *in vivo*. The evidence supporting this is that APC-depleted grafts are not rejected, even when lymphokine producing T cells, placed adjacent to an established graft, were used as the source of 'help' (92). Thus, cytokine producing CD4$^+$ T cells do not efficiently help CD8$^+$ T cells mediate graft destruction in this model.

Such CD4$^+$ T cells, specific for graft antigen in association with host MHC, could lead to either a cell-mediated DTH response or a humoral response. These are the two classes of responses shown to be regulated by cytokines. It is possible that the CD4$^+$ T cells interact with B cells and lead to the production of graft-specific antibody. This antibody is not graft destructive, as evidenced by cultured allograft survival, and may be protective. In support of this notion, serum from animals bearing long-term APC-depleted allografts contains anti-donor antibody (7) and, histologically, islet allografts from tolerant animals have cellular accumulations surrounding, but not invading the graft. This indicates that the graft is recognized by the immune system, but it is protected rather than rejected. Some grafts do have areas of invasive infiltrate but the graft is not destroyed. Thus, it appears that even if initiated, the normal immune response can be prevented from destroying the graft. These observations are indicative of dominant tolerance and, possibly, the intricate balance between immunity and tolerance in these animals.

What remains to be determined, then, is whether the tolerant state is CD4$^+$ T cell dependent and/or B cell dependent. Does graft-specific antibody play a role in the protection of the allograft or is such antibody simply a marker for a Th2-type of response? If this tolerant state is due to a particular cytokine profile produced by CD4$^+$ T cells, activated via the indirect pathway, it will be important to determine how to direct the switch from cell-mediated to humoral responses or vice versa.

Speculations of tolerance induction

The induction and maintenance of self tolerance is a complex and multifaceted process. There are few ideas that are acceptable to all investigators, however, it is unlikely that tolerance to self components expressed intrathymically and in the peripheral environment can be accounted for with one exclusive mechanism. Thus, the induction of tolerance to self components may involve a spectrum of responses ranging from a deletional mechanism to a lack of T cell activation (T cell indifference to the antigen). The mechanism operating will likely depend on the site of T cell-MHC+antigen interaction (thymus versus periphery), the stage of T cell maturation, and the nature of antigen presentation. This latter feature would include the signals received by the T cell, the type of cell presenting the antigen and cytokines in the local environment of the T cell - APC interaction. The spectrum of responses resulting in tolerance induction are illustrated in Figure V-1 below.

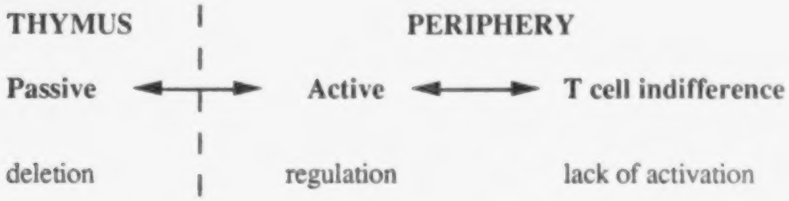

FIGURE V-1. *Speculative view of tolerance induction.* A spectrum of responses play a role in the induction of tolerance.

On one end of the spectrum are passive mechanisms such as clonal deletion (and possibly clonal anergy). T cell clonal deletion may occur only in the thymus and at immature stages of thymocyte development. Autoreactive thymocytes recognizing self MHC antigens with appropriate affinity are destined to die and will never reach the periphery. T cells with specificity for self antigens not expressed in the thymus will mature and exit the thymus. On the other end of the spectrum, opposite deletion, are T cells that are not activated to respond, that is, the T cells are indifferent to self components expressed on peripheral tissues. Although referred to as T cell indifference or ignorance, it should be noted that immune responses other than the expected autoimmune response (such as a humoral response) may be occurring. Tissue specific antigens can evade the immune system by being located in 'privileged' sites or on parenchymal S- cells which are incapable of supplying the second CoS signal necessary for T cell activation. Because the two signals required for T cell activation are not provided, T cells specific for the antigen and self MHC would remain in a naive state. In this case, TCR occupancy (signal 1) would be a null event; T cells are neither activated nor tolerized. Appropriate antigen presentation may, then, lead to an immune response. Support for this comes from APC-depleted allografts residing in the periphery of the scid mouse during T cell development or 'metastable' grafts in immunocompetent adult animals; appropriate immunization leads to graft destruction indicating that peripheral T cells were neither activated or tolerized. Similar results from Markmann *et al* (48) and Ohashi *et al* (47) have already been discussed. Also, experimental models of autoimmune disease (93-95), induced by immunization with high doses of antigen and adjuvant, indicate that normal animals have T cells which are capable of being activated under appropriate conditions. Additional support comes from observations that the release of sequestered antigens following eye injury can lead to sympathetic uveitis, a lymphocytic inflammatory response that can also attack the uninjured eye (96). Sprent has proposed a model of autoimmunity in which low numbers of T cells in an individual allow opportunistic infections and inflammation, resulting in the release of sequestered antigens to which T cells are not tolerant (97).

In between these two extremes, active regulatory mechanisms may play a role. The induction and maintenance of allograft tolerance in the adult animal would be included in this part of the spectrum. As proposed for tolerance induction to APC-depleted allografts, antigen shed from tissues, processed and presented in association with self MHC, activates $CD4^+$ T cells which function as regulatory cells, leading to protective, rather than destructive, responses. $CD4^+$ regulatory T cells may provide help for B cells leading to the production of idiotypic or anti-idiotypic antibodies, proposed to play a major role in the maintenance of self tolerance by idiotypic network theorists (98). Such a mechanism could explain why germ-free animals contain large quantities of antibody in their serum and have as many activated splenic T and B cells as normal mice (99, 100). Supposing that tolerance to allografts is maintained by regulatory $CD4^+$ T cells, activated via the indirect pathway, several predictions can be made.

1) If the indirect pathway of antigen presentation functioned to protect against autoimmune responses, it would be expected that organs expressing high levels of antigens (including MHC) on parenchymal (S^-) cells would generate more regulatory-type cells than tissues expressing (or shedding) very little antigen. Tissues expressing little antigen would be closer to the 'indifference' end of the spectrum in which T cells would essentially ignore antigen. This may explain why tissues expressing low levels of MHC (and presumably other antigens), for example, islets, neuronal tissue or erythrocytes are more apt to be targets of autoimmunity. That is, $CD4^+$ regulatory T cells may not be generated in sufficient numbers to prevent an autoimmune response from occurring when antigen is presented appropriately. It is intriguing that liver allografts, a large source of donor antigen, can spontaneously induce a tolerant state (101). This type of allograft would strongly elicit the indirect pathway.

2) If the regulation of cell-mediated and humoral responses is influenced by antigen dose (72), then tolerance induced by administration of donor antigen would also be dose-dependent. The ability of donor MHC antigen to induce tolerance is extremely dose-dependent (102).

3) Tolerance induction by regulatory T cells would be $CD4^+$ T cell dependent. Hall and colleagues (84, 103) have described $CD4^+$ T cells

responsible for the maintenance of heart allograft tolerance in models utilizing anti-donor antibody treatment or CsA. Tolerance induced by donor antigen administration, in combination with anti-CD4 mAb therapy, is dependent on the dose of mAb used, with high and low doses being less effective (104). This finding would be consistent with fluctuations between cell-mediated and humoral responses.

4) Donor-specific blood transfusions would be a good model for eliciting T cell activation via the indirect pathway. Takeuchi *et al* (81) has suggested that tolerance induced by DSBT is due to the differential activation of Th2-like T cells. The DSBT-induced tolerance model of Dallman *et al* (41) could also be explained by T cell regulation via a Th2-type cytokine profile. The tolerant state in this model can be broken *in vivo* by administration of IL-2 (105), possibly switching the class of response from humoral to cell-mediated. Also, graft-infiltrating cells, in this model, do not produce IL-2 (41). It has not been determined whether IL-4/IL-10 producing cells are present.

5). A role for the indirect pathway in tolerance induction would predict that the early administration of donor antigen to recipients of APC-depleted allografts would accelerate the induction of the tolerant state. Consistent with this prediction is the observation that administration of donor antigen, in the form of UV-irradiated spleen cells, accelerated the establishment of graft stabilization (39).

6). Cyclosporin A can switch immune responses from DTH to humoral or vice versa depending on factors such as the drug concentration or antigen dose (106). This finding may explain the observation that the clinical effectiveness of pretransplant blood transfusions has been reduced since CsA use has become widespread (107). Similarly, the ability of CsA to block RS-61443 induced tolerance to islet allografts (108) may be due to a switch in T cell regulatory responses.

7) If tolerance to self antigens expressed exclusively on extrathymic parenchymal (S^-) cells can develop via indirect processing of antigen, then it is conceivable that Th2-type 'protective' responses could be generated toward tumor-specific antigens shed from parenchymal cells. Such a scenario might explain the body's difficulty in mounting effective anti-tumor responses. In support of this idea, an early attempt to destroy tumor growth by immunization of mice with heat-killed tumor cell emulsions paradoxically enhanced tumor growth (109). More recently, North and colleagues (110) have demonstrated that, as murine tumor growth increases, $CD4^+$ T cells are progressively generated. The selective depletion of these cells leads to heightened immunity and tumor regression indicating that such $CD4^+$ T cells have a negative regulatory role in the immune response to tumor antigen. Thus, delivery of tumor antigen, in the form of S^- cells, may induce a tolerant state by the generation of a protective, rather than destructive, immune response.

The evidence supporting these predictions are suggestive of T cell regulatory responses *in vivo*. Tolerance to allografts in adult animals may be due to a balance between cells which have the potential to effect rejection and cells which can regulate their function. A clear understanding of the requirements or signals necessary for tolerance induction to alloantigens, or to self antigens in the extrathymic environment, is necessary before any application to clinical transplantation or the prevention/treatment of autoimmune disease can be made. This kind of knowledge will dictate how one should treat the allograft recipient or the patient of an autoimmune disease. For example, if allograft tolerance is due to an active process requiring the activation of donor-reactive T cells via the indirect pathway or activation of a particular subset of donor-reactive T cells, then blocking initial T cell activation may be counterproductive. The requirement for tissue immunogenicity in CsA-induced tolerance (111) or the ability of CsA to inhibit tolerance induced by RS-61443 (108) illustrates this point. If the generation of allograft tolerance requires a switch from a graft-specific Th1-like phenotype to a Th2-type phenotype, then the emphasis of future studies should be focused on how to direct or shift an immune response one way or another as in the *Leishmania* models. At the present time, the mechanism of tolerance to peripheral self antigen is not understood. Whether autoimmune disease is a failure of the clonal deletion of self-reactive T cells or the breaking of peripheral mechanisms of self-tolerance is still unknown. Studies on preventing the development of autoimmune diabetes or the recurrence of disease in models of islet transplantation by adjuvant immunotherapy (112-114) are encouraging. The normal immune response may be a constant balance between immunity and tolerance and the potential for shifting the immune response from immunity to that of tolerance is intriguing. Clarifying the nature of alloantigen presentation and the role cytokines play in the regulation of the immune response will aid in the development of future strategies for inducing transplantation tolerance in the adult.

Summary and Conclusions

Pretransplant culture of C57BL/6 pancreatic islets in 95% O_2 reduces tissue immunogenicity such that these grafts can restore euglycemia indefinitely (>100 days) in non-immunosuppressed, streptozotocin-induced diabetic BALB/c recipients. Early after grafting, such APC-depleted allografts are susceptible to rejection following host immunization with donor APCs. However, with time after transplantation, recipients progressively become resistant to APC challenge, a phenomenon referred to as graft stabilization.

1. *Graft adaptation, per se, cannot account for the graft stabilization.*

a). The long-term residence of cultured islet allografts in scid mice does not reduce the susceptibility of these grafts to rejection following host reconstitution and APC challenge.

b). BALB/c recipients of cultured B6 grafts can protect secondary donor-type sentinel islet grafts from donor-APC induced rejection.

2. *The phenomenon of graft stabilization is due to the induction of donor-specific tolerance.*

a). BALB/c recipients can protect secondary sentinel islet grafts and a proportion of donor-type thyroid grafts from rejection.

b). The tolerant state can be transferred to scid mice in a donor-specific manner.

3. *Tolerance induced in recipients of cultured islet allografts is not due to the deletion / inactivation of donor-reactive T cells. In vitro*, lymphoid cells from tolerant animals show normal anti-donor reactivity, as assessed

by: i) CTL precursor frequency,

ii) proliferative and cytotoxic responses, and

iii) lymphokine production (IL-2, IL-3, IFN-γ, and TNF).

4. *The tolerant state is not tissue (islet-peptide) specific.*

a). *In vitro* primed donor-reactive T cells secrete IL-3 in response to donor-type islet cells as antigen.

b). *In vitro* primed donor-reactive T cells are capable of mediating the destruction of established B6 islet allografts *in vivo*.

5. *T cells maturing in the presence of peripheral APC-depleted allografts are neither activated nor tolerized.* Cultured islet allografts residing in scid mice during T cell maturation are rejected following appropriate host immunization.

These results do not support the notion that the cultured islet allograft, a model of 'signal 1' antigen presentation, leads to the clonal deletion or inactivation (anergy) of either developing or mature donor-specific T cells. This implies that the two-signal model for T cell activation, as described by Lafferty (8), requires some form of negative regulation, other than signal 1 alone. Therefore, the tolerant state that develops to APC-depleted allografts in adult animals, is likely due to an active mechanism which regulates the function of donor-reactive T cells *in vivo*.

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APPENDIX A

THE THEORY OF ALLOGENEIC REACTIVITY

The following is an outline of the theory of alloreactivity as proposed by Lafferty (1). This theory is derived from the initial proposition that T cell activation is a two signal process (2). It depends on two postulates. The first postulate is that two signals, antigen and costimulator (CoS) are required for T cell activation. In its simplest form, this is expressed:

$$T \xrightarrow[(2)]{(1)} T'$$

where T is the resting T cell, T', the activated T cell and (1) and (2) represent the antigen and costimulatory signals, respectively. A corollary of this postulate is that a cell capable of providing CoS (an S^+ cell) is required for T cell activation.

The second postulate is that the release of CoS activity is regulated by a control structure, c, on the surface of the S^+ cell (3). Production and/or release of the CoS requires the interaction of 'c' with the responsive T cell. In symbolic terms:

An antigen X can be processed by a stimulator cell of 'c' genotype (${}^{c}S_{c}$).

$${}^{c}S_{c} + X \longrightarrow {}^{c}S_{c.x}$$

where ${}^{c}S_{c.x}$ is the stimulator cell expressing then processed form of antigen X in association with 'c'. A T cell is not specific for antigen X but rather sees c.x and becomes activated when the TCR engages 'c' (signal 1). This triggers the production of signal 2.

$$^{c}S_{c.x} + T_{(c.x)} \xrightarrow[(2)]{(1)} T'_{(c.x)}$$

Thus immunizing with X activates T cells of specificity $T_{(c.x)}$ and the response is MHC restricted. As such, MHC restriction is not a property of the TCR *per se*, but rather is a result of the T cell's activation requirements.

T cells of 'c' genotype do not carry receptors for c (self antigens) so the control molecule 'c' on the APC cannot be engaged. However, when a stimulator cell of 'c' genotype interacts with an allogeneic T cell of 'c_1' genotype ($^{c1}T_{(c)}$), the following reaction will occur:

$$^{c}S_{c} + {}^{c1}T_{(c)} \xrightarrow[(2)]{(1)} {}^{c1}T'_{(c)}$$

When c and c_1 are allogeneic at the MHC locus ($c \neq c_1$), $^{c1}T_{(c)}$ has a receptor for 'c' since it is not a self antigen. These responses are not MHC-restricted. This theory proposes that the antigen presenting cell (APC) plays an active role in the induction of T cells by providing both the source of antigen (signal 1) and CoS activity (signal 2). The MHC antigen on the surface of the APC (S^+) cell behaves like the control molecule.

Active versus passive antigen presentation

This theory of allogeneic reactivity implies that alloantigen can be presented in either of two distinct forms - active or passive. **Active** antigen presentation occurs when MHC antigen is presented to specific T cells on the surface of metabolically active stimulator (S^+) cells, that is, both signals required for activation are provided. **Passive** antigen presentation occurs when MHC antigen is presented on the surface of cells incapable of supplying the second (CoS) signal.

An allograft will contain resident APCs (${}^{c}S_c$) which can deliver CoS activity and activate T cells as in the above reaction. An allograft will also be composed of parenchymal cells (p_c) which express MHC antigen but cannot provide the second signal.

$$p_c \;+\; {}^{c1}T_{(c)} \xrightarrow{(1)} \text{no response}$$

Thus, an implication of this theory is that removal of active APCs from an allograft prior to transplantation would result in extended graft survival.

Indirect antigen presentation

In contrast to the direct (active) presentation of antigen, graft antigen may be shed, processed by host APCs and then presented to host T cells. In this case, exogenous antigen X would be processed and presented in association with MHC Class II of the host (${}^{c1}S_{Ia1.x}$). The host T cells will see antigen X only in association with (that is, will be restricted to) host MHC determinants.

$$X + {}^{c1}S_{Ia1} \longrightarrow {}^{c1}S_{Ia1.x}$$

$${}^{c1}S_{Ia1.x} + {}^{c1}T_{(Ia1.x)} \xrightarrow[(2)]{(1)} {}^{c1}T'_{(Ia1.x)}$$

T cells activated in this manner will be specific for (Ia1.x) which is not present on allogeneic tissue, that is, they are not graft specific (1). Such T cells can 1) interact with host B cells leading to the formation of graft-specific antibody, 2) provide a source of help for graft specific CD8$^+$ T cells and/or 3) mediate a DTH response.

SYMBOLS

c	control structure on the surface of the stimulator cell which regulates the release of CoS activity
${}^{c}S_{c.x}$	stimulator cell expressing complex of c and processed antigen, x on the cell surface
$T_{(c.x)}$	resting T cell bearing receptor for the processed antigen x in association with c
$T'(c.x)$	activated T cell with receptor for x in association with c
Pc	parenchymal cell which expresses MHC antigen on its surface but cannot supply a costimulatory signal
${}^{c1}T_{(c)}$	resting T cell of genotype c1 with receptor for allogeneic c
${}^{c1}S_{Ia1.x}$ in	stimulator cell of c1 genotype expressing processed antigen x association with Class II (Ia) MHC antigen
${}^{c1}T_{(Ia1.x)}$	T cell of c1 genotype expressing a receptor for processed antigen x in association with Class II MHC antigen

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APPENDIX B

ANTIGENICITY VERSUS IMMUNOGENICITY

EXPERIMENT: *IFN-γ treatment of high O_2 cultured islet allografts.*

RATIONALE: The classical concept of alloreactivity implies that graft 'antigenicity' plays the dominant role in allograft immunity. In contrast, graft 'immunogenicity' is the key factor in the stimulator cell model. There is some controversy over whether prolonged graft survival, as a result of tissue pretreatment, is due to the loss of stimulator cell activity or to changes in MHC antigen expression. This experiment investigated the role of MHC antigen expression in the survival of cultured islet allografts, in particular, does high O_2 culture alter islet immunogenicity or antigenicity?

EXPERIMENTAL PROTOCOL: Streptozotocin-induced diabetic CBA or BALB/c mice were grafted with 400 BALB/c or B6 islets, respectively. Islets were either untreated (immunogenic) or cultured for 7 days in an atmosphere of 95% O2 to reduce tissue immunogenicity (nonimmunogenic). In some instances, recombinant IFN-γ (300U/mL) was added during the last three days of culture. Immunohistochemical techniques indicated that Class I MHC antigen expression was markedly increased following incubation with IFN-γ. Class II MHC antigen expression was not detectable.

TABLE B-1. Effect of IFN-γ treatment on the survival of cultured islet allografts.

Group	Islet Culture	IFN-γ Treatment	Graft Survival (days)
BALB/c --> CBA I	---	---	6, 7, 9, 11,12, 14, 15, 25, 28, >100
II	+	---	9, 30, >100 (x 6)
III	+	+	17, >100 (x 5)
B6 --> BALB/c I	---	---	9, 13, 14, 14, 15, 15, 16, 17, 18, 19, 24, >100,>100
II	+	---	>100 (x 3)
III	+	+	>100 (x 3)

Diabetic mice were grafted with allogeneic islets which were either untreated or cultured in 95% O_2 for 7 days to reduce tissue immunogenicity. Where indicated, cultured islets were incubated in 300U/mL recombinant IFN-γ on days 5-7 of culture. Class I MHC expression on islet tissue was profoundly enhanced by such treatment.

CONCLUSION: These results demonstrate that high O_2 cultured islets, treated with IFN-γ to increase MHC antigen expression, survive indefinitely in allogeneic recipients. This indicates that graft immunogenicity, rather than antigenicity, is the major factor in allograft survival, as suggested by the stimulator cell model.

APPENDIX C

TISSUE SPECIFICITY OF THE TOLERANT STATE

EXPERIMENT I. *T cell subsets sufficient for thyroid allograft rejection.*

RATIONALE: The observation that a proportion of animals bearing stable islet allografts were able to reject secondary donor-type thyroid allografts (Table II-5) suggested that a tissue (islet)-specific form of tolerance may be involved. It is conceivable that such tissue-specific tolerance could be due to differences in Class I versus Class II MHC antigen expression between islets and thyroids. Tolerance to cultured islet allografts is presumably at the level of Class I MHC antigen as this tissue expresses only Class I MHC antigen. Thyroid allografts express both Class I and Class II MHC antigen so rejection could have been mediated by Class II-restricted CD4$^+$ T cells. This experiment set out to determine which T cell subsets were capable of mediating thyroid allograft rejection.

EXPERIMENTAL PROTOCOL: C57BL/6 (B6, H-2^b) thyroid lobes were grafted beneath the kidney capsule of BALB/c (H-2^d) recipients on day 0. Such recipients were untreated, or injected with depleting anti-CD4 (GK1.5) or anti-CD8 (2.43.1) monoclonal antibodies, 10 mg/kg on days -1, 0, 1 and 7 relative to grafting. The percentage of CD4$^+$ and CD8$^+$ T cell subsets in peripheral blood was determined by FACS analysis on days 7, 14, and 21.

TABLE C-1. Percentage of T cell subsets in peripheral blood of untreated or antibody-treated BALB/c (H-2^d) recipients of B6 (H-2^b) thyroid allografts.

	Untreated		Anti-CD4 treated		Anti-CD8 treated	
Day	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
7	44	10	2	30	80	0.4
14	61	9	2	38	79	0.4
21	53	11	3	26	72	0.5

BALB/c recipients were treated with depleting anti-CD4 (GK1.5) or anti-CD8 (2.43.1) monoclonal antibodies (10 mg/kg) on days -1, 0, 1 and 7, relative to transplantation of B6 (H-2^b) thyroid allografts on day 0. The percentages of CD4$^+$ and CD8$^+$ T cells in peripheral blood, analysed by flow cytometry the days indicated, are shown for representative animals.

TABLE C-2. B6 (H-2^b) thyroid allograft survival in untreated or anti-T cell antibody-treated BALB/c (H-2^d) recipients.

Group	Recipient Monoclonal Antibody Treatment	B6 Thyroid Allograft Survival
I	None	0 / 4
II	anti-CD4 (GK1.5)	1 / 3
III	anti-CD8 (2.43.1)	0 / 4

BALB/c mice were treated with depleting anti-CD4 or anti-CD8 monoclonal antibodies on days -1, 0, 1 and 7 and grafted with B6 thyroid allografts on day 0. On day 21, thyroid allograft survival was assessed by ^{125}I incorporation (cpm ≥ 5 times that of the control, non-grafted kidney) and confirmed by histological examination.

CONCLUSION: These results indicate that $CD4^+$ T cells are capable of mediating thyroid allograft rejection, and therefore, the destruction of donor-type thyroid allografts in animals bearing stable islet allografts could potentially be due entirely to CD4+ T cells. This evidence supports the hypothesis that the tolerant state is 'tissue-specific' with respect to differences in Class I and Class II MHC antigen expression on islets and thyroids. It does not, however, exclude the possibility of 'tissue-specificity' due to an islet-specific peptide.

EXPERIMENT II. *Peripheral administration of primed T cells.*

RATIONALE: T cells from tolerant animals, primed to donor antigen *in vitro*, were capable of mediating donor-type islet graft destruction when placed adjacent to an established islet allograft *in vivo* (Table III-2). This experiment did not eliminate the possibility that primed cells from tolerant animals lacked appropriate adhesion/accessory molecules, necessary to home to the graft. The preliminary experiment described below set out to determine whether *in vitro* primed T cells from tolerant animals were capable of destroying islet grafts following peripheral administration, that is, whether they were capable of homing to the graft.

EXPERIMENTAL PROTOCOL: Spleen cells from tolerant or age-matched control BALB/c animals were activated *in vitro* with either donor-type (B6) or third party (CBA) stimulator cells. Such cultures were expanded in IL-2 containing medium on day 4. On day 7, 10×10^6 BALB/c anti-B6 (d anti-b) T' or BALB/c anti-CBA (d anti-k) T' were injected into the peritoneal cavity of C.B-17scid mice bearing established B6 or CBA cultured islet allografts. Two weeks later, grafts were examined macroscopically and histologically.

TABLE C-3. Survival of cultured B6 or CBA islet grafts in scid (H-2^d) mice after i.p. injection of 'd anti-b' or 'd anti-k' T cells, respectively.

Group Number	Recipient Treatment	Islet Graft Survival B6 (H-2^b)	 CBA (H-2^k)
I	None	1 / 1	2 / 2
II	Tolerant T'	0 / 2	0 / 3
III	Control T'	1 / 1	0 / 3

Spleen cells from tolerant and control BALB/c (H-2^d) animals were activated *in vitro* with B6 or CBA stimulators. On day 7, C.B-17*scid* mice, bearing established B6 or CBA cultured islet grafts, were injected ip with 10×10^6 subcultured anti-B6 or anti-CBA primed T cells, respectively. Graft survival was assessed histologically two weeks later.

CONCLUSION: Though preliminary, these results indicate that primed T cells from tolerant animals are capable of homing to, and destroying, established donor-type islet allografts. This evidence does not support the hypothesis that the tolerance induced to cultured islet allografts is tissue-specific with respect to an islet-specific peptide.

APPENDIX E

MATERIALS AND METHODS

Animals. Six to 8 week old male C57BL/6ByJ (B6, H-2^b), CBA/J (CBA, H-2^k) and BALB/cByJ (BALB/c, H-2^d) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). C.B-17 *scid/scid* (scid, H-2^d) mice were generously provided by L. Shultz and bred at the Barbara Davis Center.

Cell Lines. P815, a DBA/2 (H-2^d) mastocytoma, EL-4, a C57BL/6N (H-2^b) lymphoma, and R1.1, derived from a C58/J thymoma (H-2^k) were used as targets in cytotoxicity assays or as antigen in lymphokine triggering assays. These cell lines were maintained by serial passage in Dulbecco's modified Eagle's minimal essential medium (DMEM, Gibco Laboratories, Grand Island, NY) supplemented with 44mM sodium bicarbonate (Fisher Scientific, Fair Lawn, New Jersey), 20mM HEPES buffer (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Research Organics, Inc., Cleveland, Ohio). Prior to use, DMEM was supplemented with 10% heat-inactivated fetal bovine serum (FCS, Gibco) and antibiotics (5mg penicillin, 5mg streptomycin and 10mg neomycin per 100mL, PSN, Gibco). The same culture conditions were used for WEHI-164 cells, a TNF-sensitive line (1) used in bioassays for TNF. FDC-P1 (FD) cells (2), used in a bioassay for IL-3 detection, are dependent on hematopoietic growth factors (IL-3) and were maintained as outlined above with the addition of WEHI-3 (3) (IL-3 containing) SN. The GK1.5 hybridoma secretes a rat anti-mouse L3/T4 (anti-CD4) monoclonal antibody (isotype IgG_{2b}) (4). Anti-CD4 culture supernatants and ascites were derived from this cell line, maintained as above. Similarly, rat anti-mouse CD8 monoclonal antibodies were obtained from the 2.43.1 hybridoma (5). The monoclonal antibody produced by this hybridoma is specific for CD8.2 and is the IgG_{2b} isotype. HO-13-4 cells, maintained in DMEM with 10% FCS, produce an antibody reactive to the T cell Thy-1.2 antigen (6). SN from these cells was used for T cell depletion of bone marrow and spleen cells.

Islet Isolation. Four to 12 pancreatic donors (6-12 weeks of age) were pretreated with 10 µL/g cyclophosphamide (Cytoxan, Nenar, Adria Labs Inc., Columbus, OH) on days -4 and -2 prior to organ removal. On the day of isolation, 30 min prior to pancreas removal, mice were injected ip with 40mg pilocarpine hydrochloride (2%, Isoptocarpine, Alcon, Puerto Rico) /kg body weight.

Medium A consisted of Hanks Balanced Salt Solution (Gibco, Grand Island, NY) buffered with 20mM HEPES (Calbiochem, La Jolla, CA) (HBSS) and containing 0.1% bovine serum albumin (BSA, 15% in HBSS) and 0.1mg deoxyribonuclease I (DNase)/mL (Sigma, type 1, St. Louis, MO). Collagenase, 7.5mg, (*Clostridium histolyticum*, Boehringer Mannheim, Indianapolis, IN) was dissolved in 5mL of medium (1.5 mg/mL) and filtered into siliconized glass vials. Four pancreata were placed per vial and were shaken in a 37°C water bath (Eberbach, Ann Arbor, MI) for 20 min at 250 cycles/min. Collagenase action was stopped by the addition of cold medium. The tissue was allowed to settle for 5 min before removing the supernatant. Collagenase (5 mL at 0.75 mg/mL) was added and the suspension shaken in the water bath for 5 min, stopping the action of collagenase with cold medium B (HBSS with 10% FCS and PSN). Supernatants were removed after 5 min. These 5 min collagenase digestions and washes were repeated two times. The pancreatic digest was placed in Falcon petri dishes and, with the aid of a dissecting microscope, undigested tissue was removed for a final incubation with collagenase. Lymph nodes and large ductal tissue were removed. The pancreatic digest (from 8-12 pancreata) was transferred to a 50mL polystyrene conical centrifuge tube (Corning) and centrifuged at 75g for 60 seconds in an IEC Clinical table top centrifuge (International Equipment Co., Needham Heights, Mass). The pellet was resuspended in 5mL of 25% Ficoll (Sigma Type DL-400). Three mL aliquots of 23%, 20% and 11% Ficoll were carefully overlayed. Prior to use, the density of Ficoll was adjusted to values of 1.095, 1.085, 1.075 and 1.045, respectively, using a DMA 35 digital densitometer (Anton Parr, Graz, Austria). The Ficoll gradient was spun in a Beckman Model TJ-6 tabletop centrifuge at 550g for 20 min. Isolated islets were removed from the 23-20% interface and washed several times in medium B to remove Ficoll and exocrine contaminants. Islets were then placed in a petri dish (Falcon, Becton Dickinson Labware, Lincoln Park, New Jersey) and hand-picked free of contaminating debris with the aid of a dissecting stereomicroscope and a finely-drawn siliconized Pasteur pipette. Freshly isolated islets were transplanted or prepared for high O_2 culture.

Islet Culture. Hand-picked islets were prepared for high O_2 culture by placing 50 islets in individual wells of a 96-well round bottom hydrophobic microtitration plate (Linbro®, Flow Laboratories, McLean, Virginia). Medium B supernatants in each well were removed and 50μL of RPMI 1640 with 10% FCS and antibiotics was added to each well. To promote aggregation, culture plates were spun at 135g for 4 min in a

Beckman centrifuge and were placed in humidified gastight chambers containing 95% O_2 and 5% CO_2 at atmospheric pressure. The chambers were kept in a 37°C dry air incubator (Forma Scientific, Marietta, Ohio).

After 3 days of culture, aggregated islet clusters were transferred to 36mm 6-well flat bottom hydrophobic plates (Falcon). Each well contained two islet clusters in 0.75 mL of supplemented RPMI culture medium. These plates were maintained in chambers as above for an additional four days. Culture medium was changed on day 5 and islets were grafted on day 7.

Induction of Diabetes. Streptozotocin (SZ, 250mg, Calbiochem - Behring Corp., La Jolla, CA) was dissolved in 0.1M acetate buffer (pH 4.4) consisting of 0.2M acetic acid and 0.2M sodium acetate. SZ was injected via the retro-orbital sinus of anesthetized mice at doses dependent on the mouse strain. BALB/c and CBA mice received 225 and 275 mg SZ/kg body weight, respectively. C.B-17 *scid* mice received 225 mg SZ/kg body weight ip, rather than iv. Non-fasting whole blood glucose was routinely measured using an Exactech® blood glucose meter (MediSense, Inc., Cambridge, MA). A minimum of two consecutive blood glucose values ≥ 20mM was indicative of the induction of diabetes and candidates for transplantation.

Transplantation. Islet recipients were anesthetized with Metofane (Methoxyflurane, Pitman-Moore, Inc., Mundelein, IL) and the left kidney was exposed through a small flank incision. Ensuring the kidney was moistened with HBSS, a nick was made in the renal capsule at the posterior end of the kidney using a 26G needle. A blunt-ended glass probe was inserted beneath the capsule to separate it from the kidney parenchyma. Using a Fisherbrand microhematocrit capillary tube and microcap bulb (Microcaps, Drummond Scientific, Broomall, PA), islet clusters were picked up and inserted beneath the capsule at the anterior end of the kidney. The kidney was placed inside the body cavity and the incision closed with a MikRon 9mm autoclip (Clay Adams, Becton Dickinson, Parsippany, NJ).

Secondary thyroid grafting in recipients of stable allografts. Animals bearing stable islet allografts received untreated donor-type (B6) and third-party (CBA) thyroid allografts at opposite poles of the kidney contralateral to the islet graft. Thirty days later, thyroid function was assessed by their ability to incorporate ^{125}Sodium iodine (^{125}I, NEN® Research Products, Du Pont, Boston, MA). Animals were injected with 0.25 μCi of ^{125}I ip and, after 30 days, incorporation from the thyroid grafts and control (islet-grafted) kidneys were determined on a Micromedic ME *plus* automatic gamma counter (Micromedic Systems, Inc.). Thyroid allograft survival was defined as ^{125}I incorporation $\geq$ 5-fold the incorporation of the islet-grafted or non-grafted kidney.

IN VITRO ASSAYS

Mixed Lymphocyte Culture (MLC).

A. Preparations of stimulators: Mouse spleen single cell suspensions were homogenized with a sterile Pyrex tissue grinder (Corning Inc., New York) with 3-5mL of HBSS and PSN and placed in a 15mL conical centrifuge tube (Corning) for 5 min. Homogenate supernate was transferred to another 15 mL conical tube and centrifuged for 5 min at 250g. The supernate was discarded and cells were resuspended in 10 mL of Eagle's minimal essential medium (EMEM, Gibco) supplemented with 20mM HEPES buffer and 26mM sodium bicarbonate. Prior to use, 0.1% (10^{-5} M) 2-mercaptoethanol (2-ME) was added. Stimulator spleen cell suspensions were irradiated with a cobalt gamma source at 2000R. Trypan blue excluding live cells were quantitated with a Reichert Bright-Line hemacytometer (Buffalo, NY) and adjusted to $3x10^6$ cells/mL.

B. Preparations of Responders: Responder cell populations were prepared from homogenates of axillary, brachial and mesenteric LN or from spleen cell homogenates prepared as described above. Responder populations were counted and adjusted to $2x10^6$ cells/mL.

C. Culture: One mL aliquots of irradiated spleen cell stimulators, mixed with 1 mL aliquots of allogeneic LN or spleen cell responders in a Falcon (Becton Dickinson Labware, Lincoln Park, NJ) 24-well culture plate, were incubated in a 10% CO_2 incubator at 37°C. To measure proliferative responses, 100 μL aliquots of responders ($2x10^5$ cells) and graded numbers of irradiated stimulators were mixed in 96-well flat-bottom tissue culture plates (Linbro®, Flow Laboratories Inc., McLean, Virginia). On days 3, 4, and 5 of primary culture, each reaction well was pulsed with 1.25 μCi (50 μL) of [^{3}H]-thymidine (NEN® Research Products, DuPont, Boston, MA) for 6h. Plates were harvested on a Skatron automatic cell harvester using printed Betaplate glass fiber filtermats (Pharmacia, Turku, Finland). [^{3}H]-thymidine was counted on an LKB 1205 Betaplate liquid scintillation counter (Pharmacia, Turku, Finland). The mean of quadruplicate samples was determined for each group. Background cpm were obtained from wells containing responders in medium alone.

Limiting Dilution Analysis. Cytotoxic T cell precursor (CTLp) frequencies were determined as follows: Limiting numbers of LN or spleen cells (32 replicates/group) from tolerant or age-matched control animals were cultured in 96-well flat-bottom microtiter plates (Linbro) with 0.5x 10^6 irradiated B6 spleen cells and Con A SN (1/60 final dilution). On days 4 and 6, 100μL of medium was removed from each well and fresh Con A SN was added. On day 7, 100μL of cells from each well were assayed for cytotoxic activity in the ^{51}Cr release assay described below. Cultures with less than or equal to 3SD of lytic activity over spontaneous release were scored negative. CTLp frequency was determined using the Poisson distribution (7). Briefly, the log of the percent negative wells was plotted against the number of cells per well. The CTLp frequency was the reciprocal of cells/well when 37% of wells were negative.

Cytotoxic T Lymphocyte Assay. CTL activity was measured in a ^{51}Chromium (^{51}Cr) release assay on day 5 of the primary MLC.

A. Preparation of Targets: 2x10^6 P815 (H-2^d), EL-4 (H-2^b) or R1.1 (H-2^k) tumor cell targets were centrifuged for 5 min at 250g. The supernate was suctioned off and the pellet resuspended in 100μL of FCS. Eighty μCi of ^{51}Cr sulfate (Amersham, Arlington Heights, IL) per 2x10^6 cells was added. After incubating for 60 min at 37°C, cells were washed three times in HBSS with 20% FCS and resuspended in one mL EMEM medium. Trypan blue excluding cells were counted and then diluted to 1x10^5 cells/mL in EMEM culture medium.

B. Preparation of Effectors: T cell blasts (T') were harvested from the MLC, washed twice in HBSS and resuspended in EMEM culture medium. Cells were counted and adjusted to 1x10^6 cells/mL. Aliquots (200 μL) of effector cells were placed in triplicate or quadruplicate wells in 96-well V-bottom tissue culture plates (Dynatech Laboratories). Serial dilutions were made in culture medium. Targets (100μL) were added to these wells and to wells containing medium only (spontaneous release) and to 1% sodium dodecyl sulfate (SDS, maximum release). Plates were incubated in a 10% CO_2 incubator at 37°C. After 4h, plates were centrifuged for 5 min at 135g to pellet cellular debris. Aliquots from each well (100μL) were transferred to 6x50mm borosilicate glass culture tubes (Kimball) and ^{51}Cr release was measured on the Micromedic gamma counter described above. Cytotoxic units (CU) per culture were determined as described by Woolnough and Lafferty (8) where one CU is the activity required to lyse one target cell under the conditions of the assay.

Lymphokine triggering. MLC cultures were expanded in 30mL of medium (EMEM plus 10% FCS and antibiotics) containing Con A SN (1/60) on day 4 of culture. Three days later, activated blasts (T') were washed several times in HBSS to remove any trace of Con A SN. These cells were counted and diluted to $2x10^6$/mL. Tumor cells of appropriate haplotype were placed in petri dishes (Falcon) and UV-irradiated for 4 min using a UV-lamp (Gates, Co., New York, NY) set at 960 μWatts UV-A/cm^2 with a Blak-Ray® short-wave ultraviolet meter (Ultra-Violet Products Inc., San Gabriel, CA). Irradiated cells were counted, spun for 5 min at 250g and resuspended to 8 or $4x10^6$ cells/mL according to the particular assay.

In 96-well flat-bottom plates (Linbro®), 200 μL of UV-irradiated tumor cells were added to duplicate wells. Two-fold dilutions of the antigen were made in culture medium (final volume of 100 μL). T' cells at

2×10^6/mL were added to each well and the plates were incubated at 37°C in a 10% CO_2 incubator. After 6h, 100µL of supernate was removed from each reaction well and placed in an adjacent well. The plates were frozen and lymphokine assays were performed at later dates. Negative controls for these experiments consisted of T' cells incubated in medium without added tumor cell antigen.

TCGF (IL-2) Assay. The IL-2 assay detects T cell growth factor in sample supernates by its ability to stimulate proliferation of Con A blasts. *Preparation of Con A Blasts:* CBA spleen cell suspensions were prepared by tissue homogenizing with HBSS and 1% PSN as described above. The homogenate pellet resuspended in 10mL of culture medium (EMEM + 10% FCS + 2-ME and PSN), counted with trypan blue and adjusted to 2×10^6 cells/mL. One mL of culture medium containing 2µg of Con A (Sigma) per mL was placed in each well of a tissue culture pretreated 24-well plate (Falcon 3008). Then one mL of the spleen cell suspension was added per well (final concentration of Con A at 1 µg/mL). Spleen cell cultures were incubated at 37°C in 10% CO_2 for four days.

Con A blasts were harvested on day 4 and washed several times to remove residual Con A. These cells were resuspended in culture medium and diluted to 4×10^5 cells/mL. In a 96-well flat-bottom microtiter plate, 100µL aliquots of samples to be assayed were serially diluted in 50µL of culture medium. The Con A blast suspension (2×10^4 cells/50µL) was added to each well and incubated in 10% CO2 at 37°C. Con A SN served as a positive control. Approximately 18h later, 50µL of [^{3}H]-thymidine at 25 µCi/mL (1.25µCi) (DuPont) were added to each well and incubated for an additional five hours. Background values were obtained from Con A blasts incubated in medium alone.

[^{3}H]-thymidine incorporation was determined as described above. Endpoint titers were assessed from a plot of log cpm versus log antigen concentration with the mean value of Con A blasts alone plus 3 SD as background.

IL-3 Assay. The IL-3 assay detects the presence of hematopoietic growth factors by their ability to stimulate proliferation of FDC-P1 (FD) cells, a bone-marrow derived hematopoietic growth factor (IL-3) dependent cell line. Log-phase FD cells were washed three times in HBSS+PSN to remove traces of WEHI-3 SN. Cells were counted and adjusted to 4×10^5 cells/mL in culture medium (DMEM plus 2.5% FCS). In 96-well flat-bottomed plates (Linbro®), 100µL aliquots of the supernatants to be assayed were serially diluted in culture medium (50µL) and 2×10^4 FD cells (50µL)/well were added. WEHI-3 SN was used as a positive control, FD cells in medium alone served as the negative control and the 'blank' well contained 100µL of medium only. Duplicate or triplicate plates were incubated for 24h. Proliferation was then assayed using an MTT (3-4,5-Dimethylthiazol-2-yl-2,5-diphenyl-tetrazoliumbromide,Sigma) colorimetric method (9). MTT (50µg in 25µL) was added to each well and the plates were incubated for an additional 2h at 37°C. Isopropanol containing 0.04N HCl (125 µL/well) was added and mixed vigorously with a multi-pipettor until all formazan crystals were dissolved. The optical density of each well was determined on a Dynatech MR 600 microplate reader (Alexandria, Virginia) using a reference wavelength of 630nm and a test wavelength of 570nm. The lower limit of detection was determined from the mean value of FD cells in medium alone plus 3 SD.

TNF Assay. Test SN was assessed for TNF activity by the ability to kill a TNF-sensitive tumor cell line, WEHI-164. Linbro® 96-well flat-bottom plates were set up as described for the IL-3 assay. Cells (5000/well) were incubated in dilutions of SN for 48h after which cell viability was assessed by the MTT colorimetric assay described above. WEHI-164 cells cultured in dilutions of Con A SN provided the positive control for TNF. Optical density readings of cells incubated in medium without test supernates (negative control) plus 3 SD provided the upper limit of the assay from which endpoint titers were determined.

Interferon-γ Assay. A solid phase enzyme immunoassay (Genzyme mouse interferon-γ ELISA, Genzyme Corporation, Boston, MA) was used to detect IFN-γ in test SN. Optical density readings were obtained using a Dynatech MR 600 microplate reader. The concentration of IFN-γ in each sample was estimated from a standard curve. The lower limit of detection of this assay was 0.2 ng/mL.

Trypsin digestion of islets. When islets were used as antigen for lymphokine triggering assays, single cell suspensions were prepared as described by Haskins *et al* (10). Handpicked B6 islets were incubated at 37°C in medium containing 2 µg/mL DNase and 0.05% trypsin (Sigma) for a total of 30 min, during which islets were vigorously resuspended after 15 min. The single islet cell suspension was centrifuged at 20g for 10 min through underlayed medium containing 4% bovine serum albumin (Sigma).

The islet pellet was resuspended in culture medium, viable cells were counted and then frozen in medium containing 15% DMSO and 30% FCS. Single cell preparations were thawed and pooled, and UV-irradiated before use in lymphokine triggering assays.

T' Transfer. *In vitro* activated T cells (T') from tolerant and control mice were clonally expanded in media containing Con A SN. On day 7 after primary MLC, 10^6 T' cells were embedded in a recipient-derived blood clot and implanted adjacent to an established sentinel B6 or BALB/c islet grafts residing in a normal BALB/c for 1-3 weeks. Grafts were examined two weeks later. In preliminary experiments, 10×10^6 T cells activated with B6 or CBA stimulators and expanded *in vitro* were injected ip into C.B-17*scid* mice bearing cultured B6 or CBA sentinel grafts.

Bone marrow reconstitution of C.B-17scid mice. Bone marrow was obtained from BALB/c or B6 mice by freeing femurs and tibias from muscle and connective tissue, removing one end of the bone, inserting a 25G needle into the bone and then flushing 3-6mL of HBSS through into a 50mL conical centrifuge tube. Cellular clumps were allowed to settle and after 5 min, the SN was removed, spun and resuspended in culture medium (EMEM with 10% FCS, 0.1mg DNase/mL and PSN). T cells ($\leq 5 \times 10^6$/mL) were depleted by incubating cells in anti-thy1.2 HO-13-4 culture SN (1/4 final dilution) and complement (Low-Tox®-M rabbit complement, 1/30 final dilution) for 1h at 37°C. Cells were washed three times in DNase containing medium, counted and resuspended to 12.5×10^6 cells/mL. 5×10^6 T cell-depleted BALB/c bone marrow cells in 0.4mL were injected into the tail vein of scid mice. Some mice received both BALB/c and B6 bone marrow at a 1:3 ratio; $5x10^6$ BALB/c and $15x10^6$ B6 cells in a total of 0.4mL. Bone marrow cells before and after T cell depletion were analyzed for T and B cell markers by FACS analysis.

Assessment of tolerance in C.B-17scid mice. Diabetic C.B-17*scid* mice were grafted with 450 cultured B6 islets and reconstituted, after two weeks, with T cell-depleted BALB/c or BALB/c plus B6 bone marrow. Seventy days after bone marrow grafting, recipient peripheral blood was assessed for T and B cell markers by FACS analysis and mice were immunized with 10^6 B6 spleen cells, depleted of T cells as described above.

Flow cytometry. All antibodies were directly conjugated to FITC. FITC-rat anti-mouse L3/T4 (anti-CD4, Clone YTS 191.1) was obtained from Caltag Laboratories, Inc., San Francisco, CA and used at 0.5 µg/sample. FITC-rat anti-mouse Lyt-2 (anti-CD8, Clone 53-6.7, 1.25 µg/sample) was from Becton Dickinson, Mountain View, CA. FITC-goat anti-mouse Ig (IgG + IgM, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was diluted to 0.8 µg/sample. FITC-rat anti-mouse Ly-5 (anti-B220, Clone RA3-6B2, Pharmingen, San Diego, CA) was used at 5 µg/sample. FITC conjugate hamster anti-mouse CD3ε monoclonal antibody (Clone 145-2C11) was obtained from Pharmingen and used at 1µg/sample.

Peripheral blood (200-400µL) was layered over 600µL of Lympholyte M Ficoll (Cedarlane Laboratories, Hornby, Canada) and spun at 400g for 10 min. Cells at the interface were collected and washed in staining buffer (PBS with 1% FCS and 2mg/mL sodium azide). Peripheral blood or spleen and LN cell populations were suspended in staining buffer and transferred to 96-well round bottom plates (Linbro®) (approximately 10^6 cells in 200µL aliquots). Plates were centrifuged at 135g for 5 min and supernates were discarded. Samples were incubated with 100µL of buffer or FITC-conjugated antibodies for 15 min at 4°C. After three 200µL washes with buffer, cells were resuspended in 500µL of buffer for flow cytometry. Frequency determinations were calculated from single-parameter fluorescence histograms on an EPICS C flow cytometer Model No 2985, Coulter Electronics, Hialeah, FL.

Histological examination of grafted tissues. Graft-bearing kidneys were removed and fixed in 10% formal saline. Paraffin sections were stained with hematoxylin-eosin and with aldehyde fuchsin to stain insulin-containing granules. Tissue sections were examined to determine the degree of tissue damage and mononuclear cell infiltration of the graft.

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